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(54) **THYROID HORMONE ANALOGS AND METHODS OF USE**

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See application file for complete search history.

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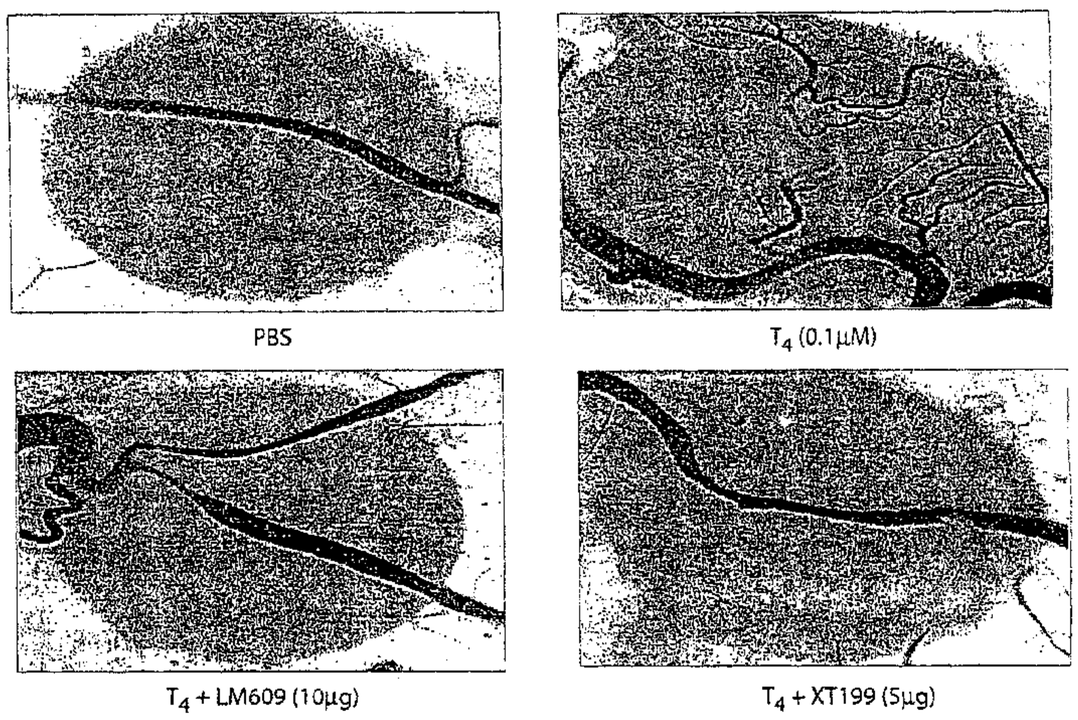
(57) **ABSTRACT**

Disclosed are methods of treating subjects having conditions related to angiogenesis including administering an effective amount of a polymeric form of thyroid hormone, or an antagonist thereof, to promote or inhibit angiogenesis in the subject. Compositions of the polymeric forms of thyroid hormone, or thyroid hormone analogs, are also disclosed.

4 Claims, 36 Drawing Sheets

Inhibitory Effect of αvβ3 MAB (LM609) and XT 199 on T4-induced angiogenesis in the CAM Model



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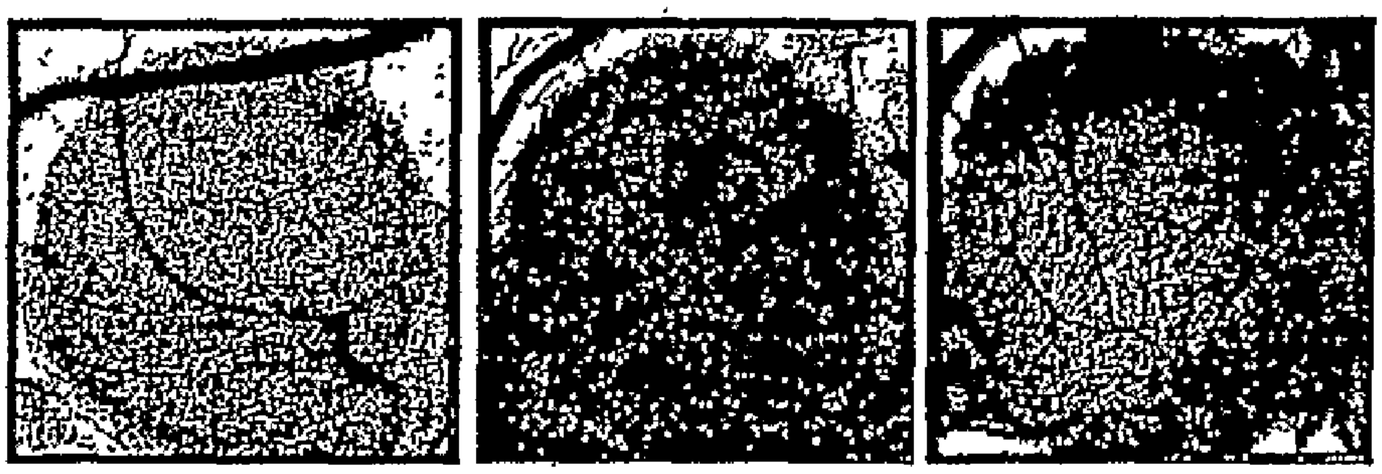


Fig. 1A

T_4 and T_3 stimulate angiogenesis in the chorioallantoic membrane model

Treatment	Angiogenesis Index
PBS	63 10
T_3 (1 nM)	121 18**
T_4 (0.1 μM)	155 11**

Fig. 1B

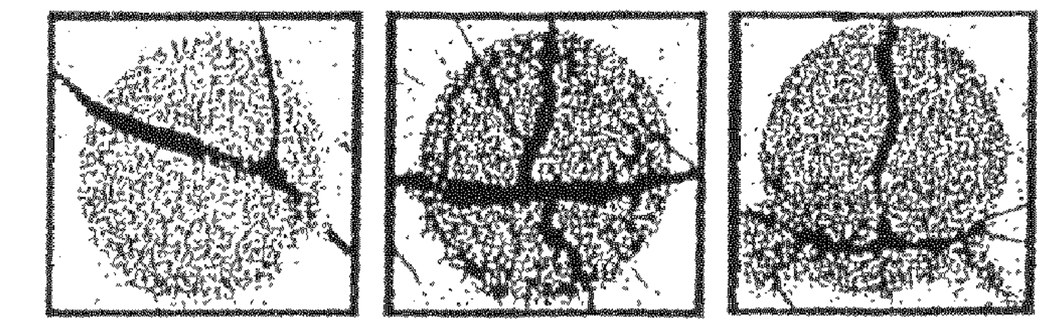


Fig. 2A

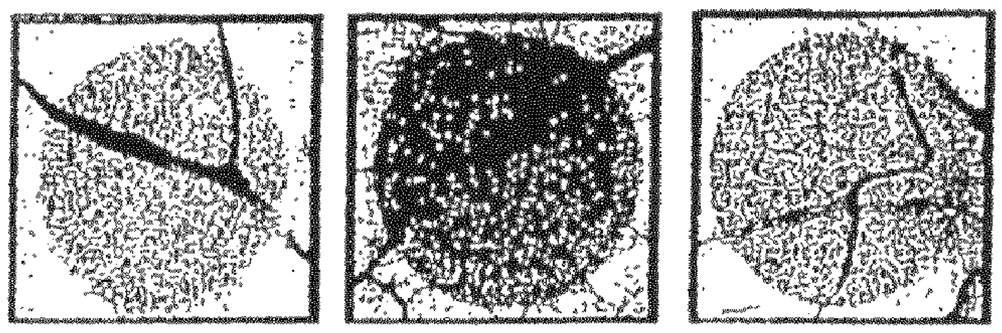


Fig. 2B

Summary of effects of T_4 and T_3 -agarose and tetrac on angiogenesis

Treatment	Angiogenesis Index
PBS	67 9
T_4 (0.1 μM)	156 16**
Tetrac (0.1 μM)	76 9
T_4 + tetrac	66 6
T_4 -agarose (0.1 μM)	194 28**
T_4 -agarose + tetrac	74 7

Fig. 2C

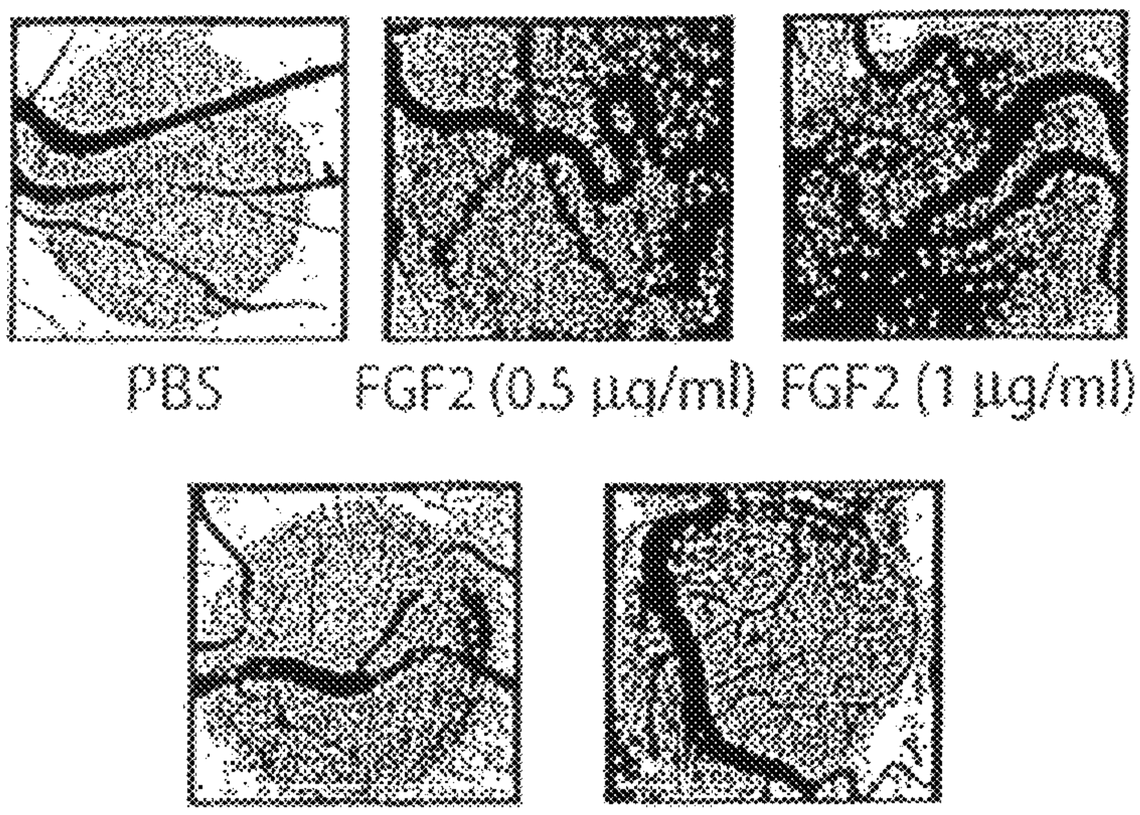


Fig. 3A

Effects of FGF2 and T_4 on angiogenesis

Treatment	Angiogenesis Index
PBS	86 11
FGF2 (0.5 µg/ml)	126 17*
FGF2 (1.0 µg/ml)	172 9**
T_4 (0.5 µM)	115 4*
T_4 + FGF2 (0.5 µg/ml)	167 10**

Fig. 3B

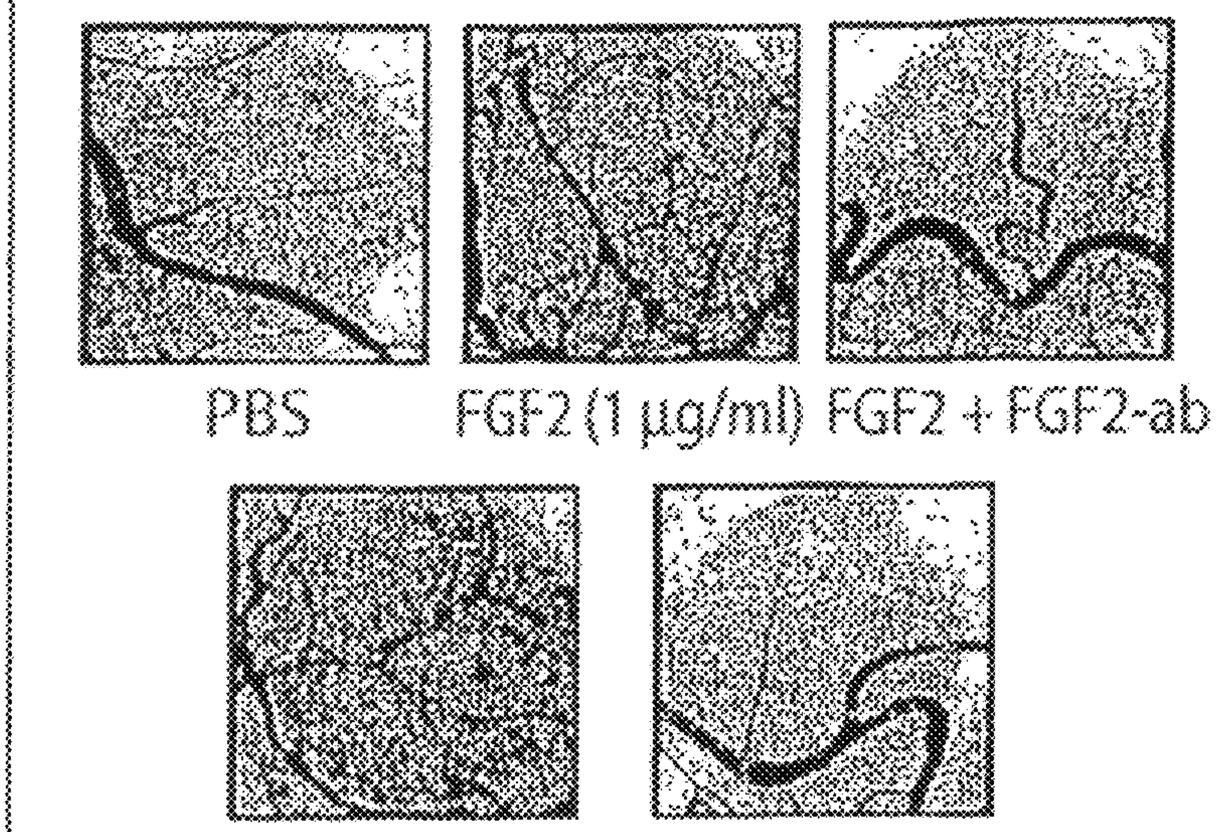


Fig. 4A

Effects of FGF2 antibody on angiogenesis stimulated by T_4 and FGF2

Treatment	Angiogenesis Index
PBS	92 10
FGF2 (1.0 µg/ml)	187 17*
FGF2 + FGF2-ab	118 7
T_4 (0.1 µM)	142 12*
T_4 + FGF2-ab	96 10

Fig. 4B

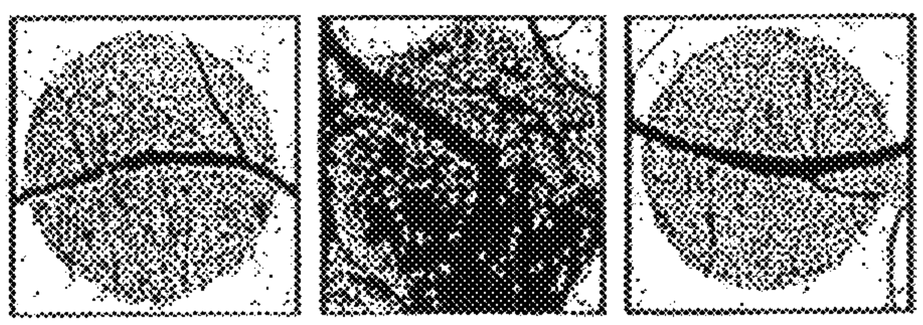


Fig. 5A

Fig. 5B

Effects of PD 98059 on angiogenesis stimulated by T_4 and FGF2

Treatment	Angiogenesis Index
PBS	63 10
T_3 (1 nM) + T_4 (0.1 µM)	153 15*
T_3 + T_4 + PD 98059 (3 µM)	50 10
PBS	86 11
FGF2 (1 µg/ml)	191 15**
FGF2 + PD 98059 (3 µM)	110 16

Fig. 5C

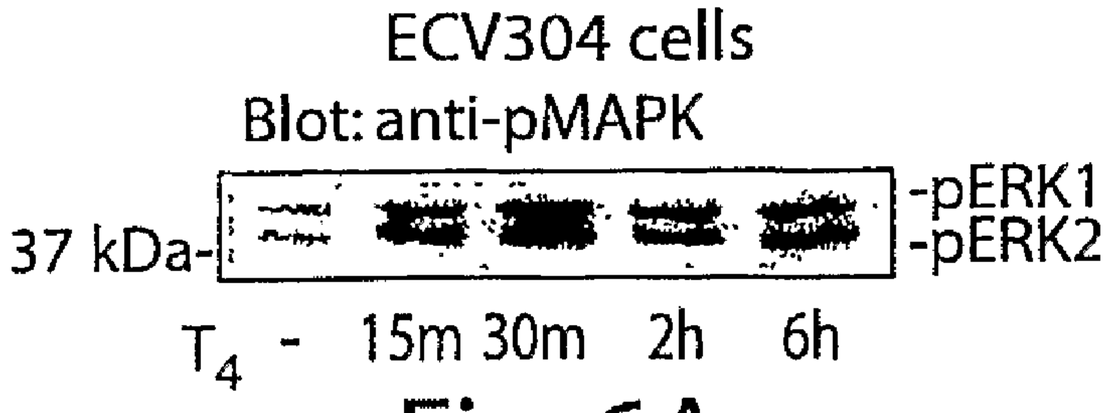


Fig. 6A

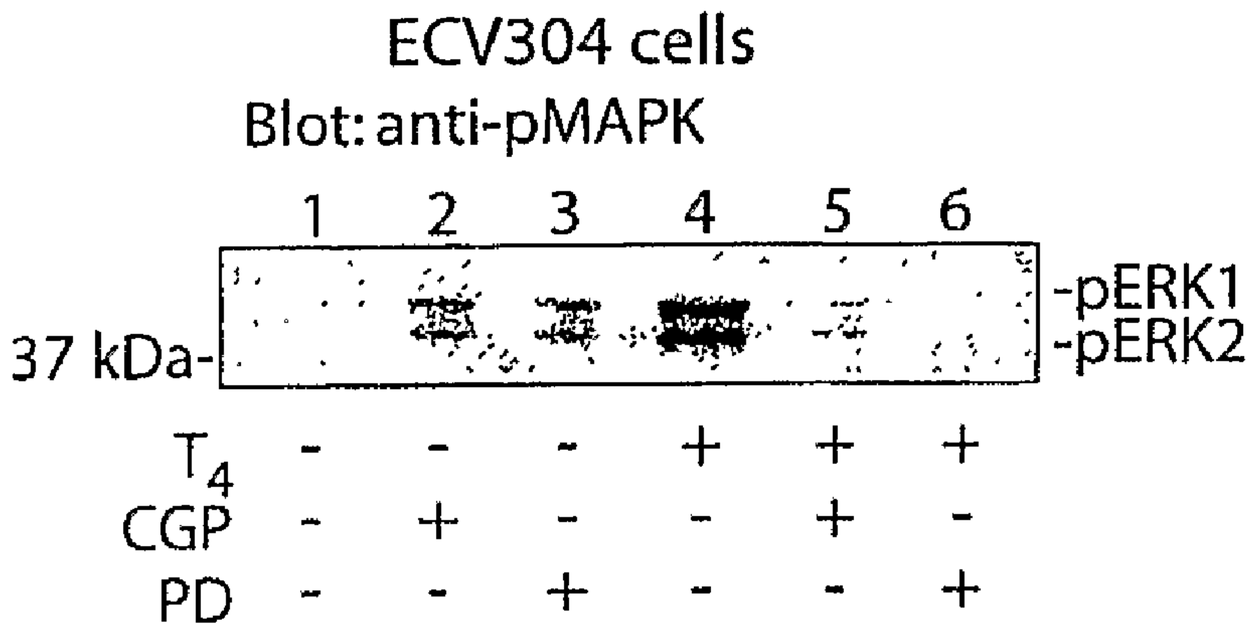


Fig. 6B

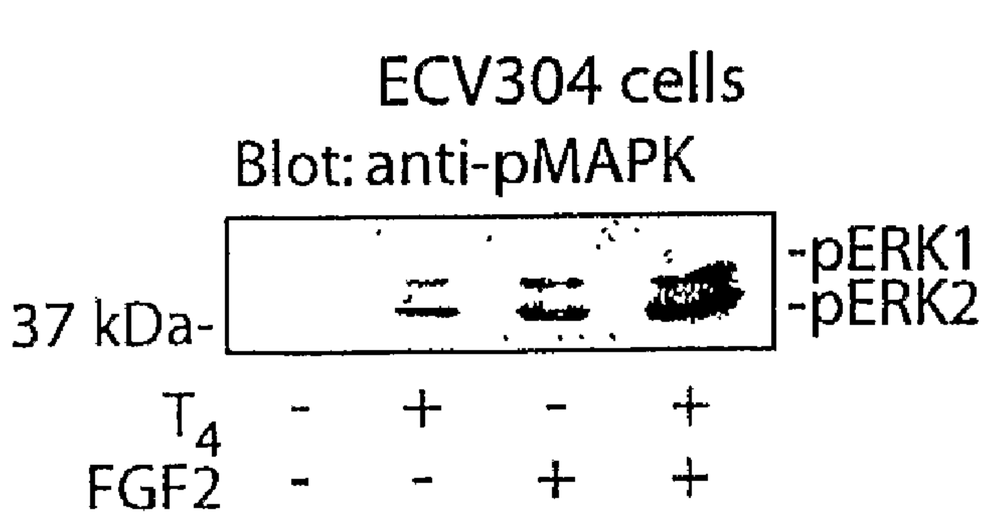


Fig. 6C

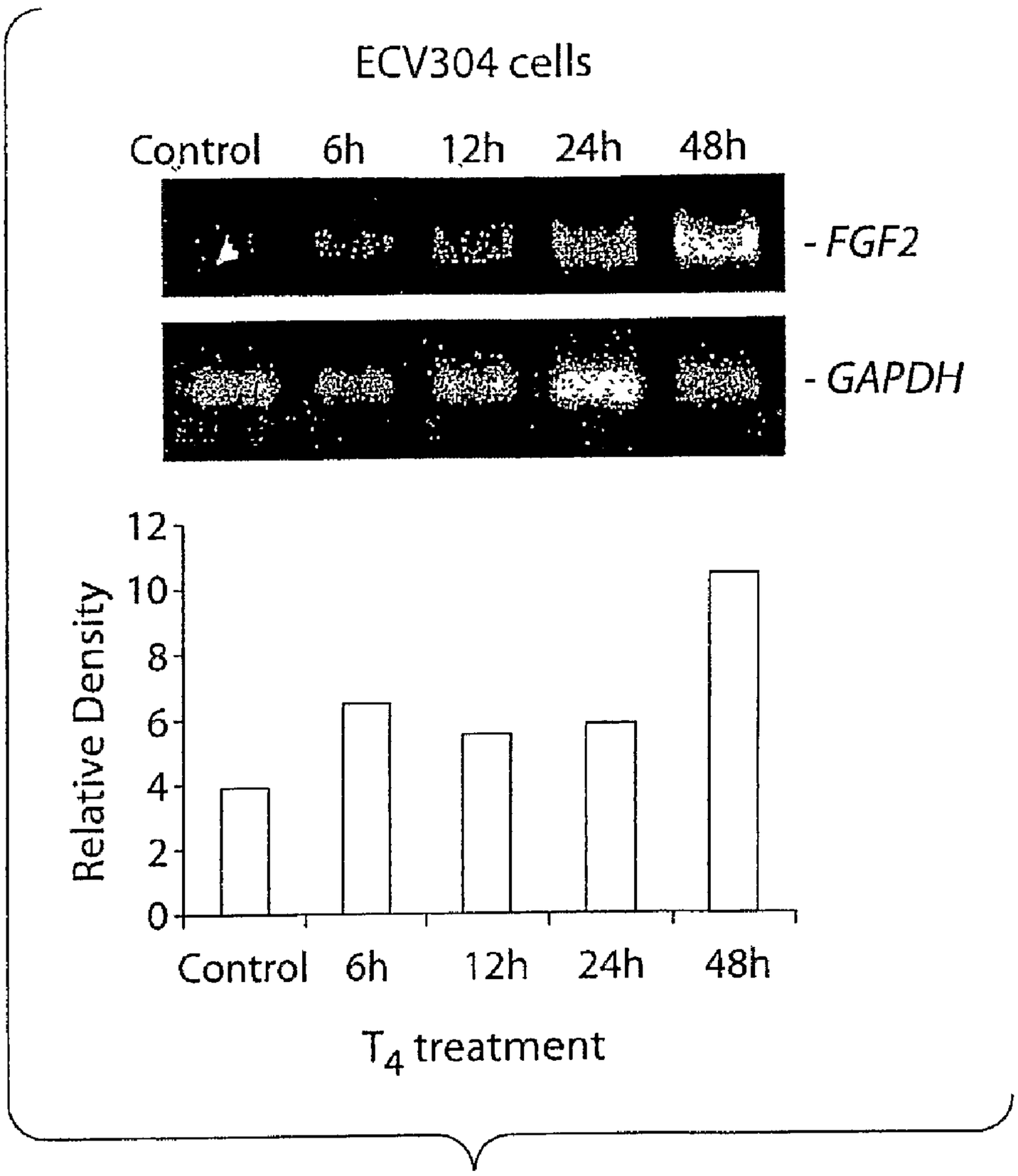

ECV304 cells
Control
6h
12h
24h
48h
- FGF2
- GAPDH
Relative Density
0
2
4
6
8
10
12
Control
6h
12h
24h
48h
T4 treatment

Fig. 7

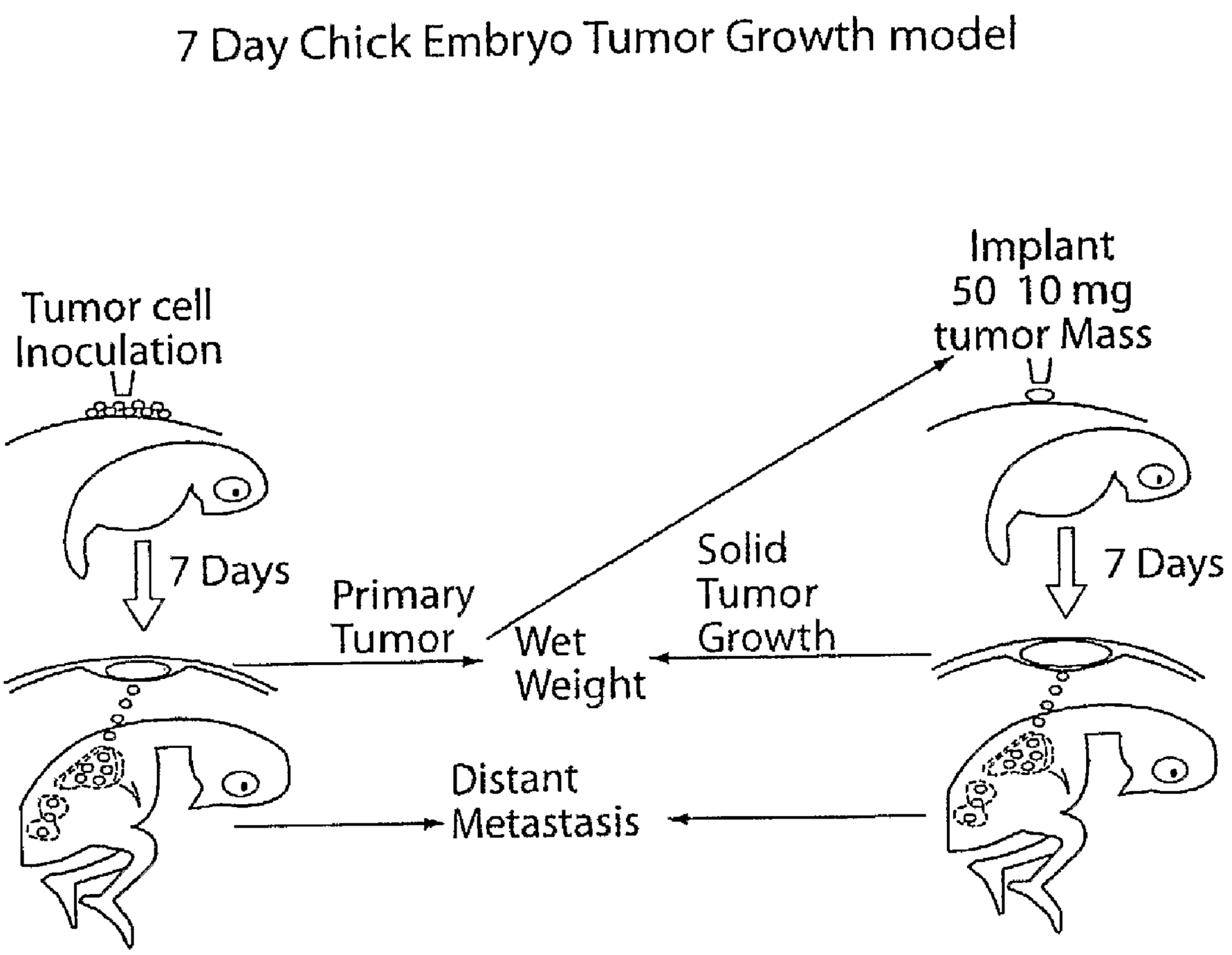

7 Day Chick Embryo Tumor Growth model
Tumor cell Inoculation
7 Days
Primary Tumor
Wet Weight
Distant Metastasis
Implant 50 10 mg tumor Mass
7 Days
Solid Tumor Growth

Fig. 8

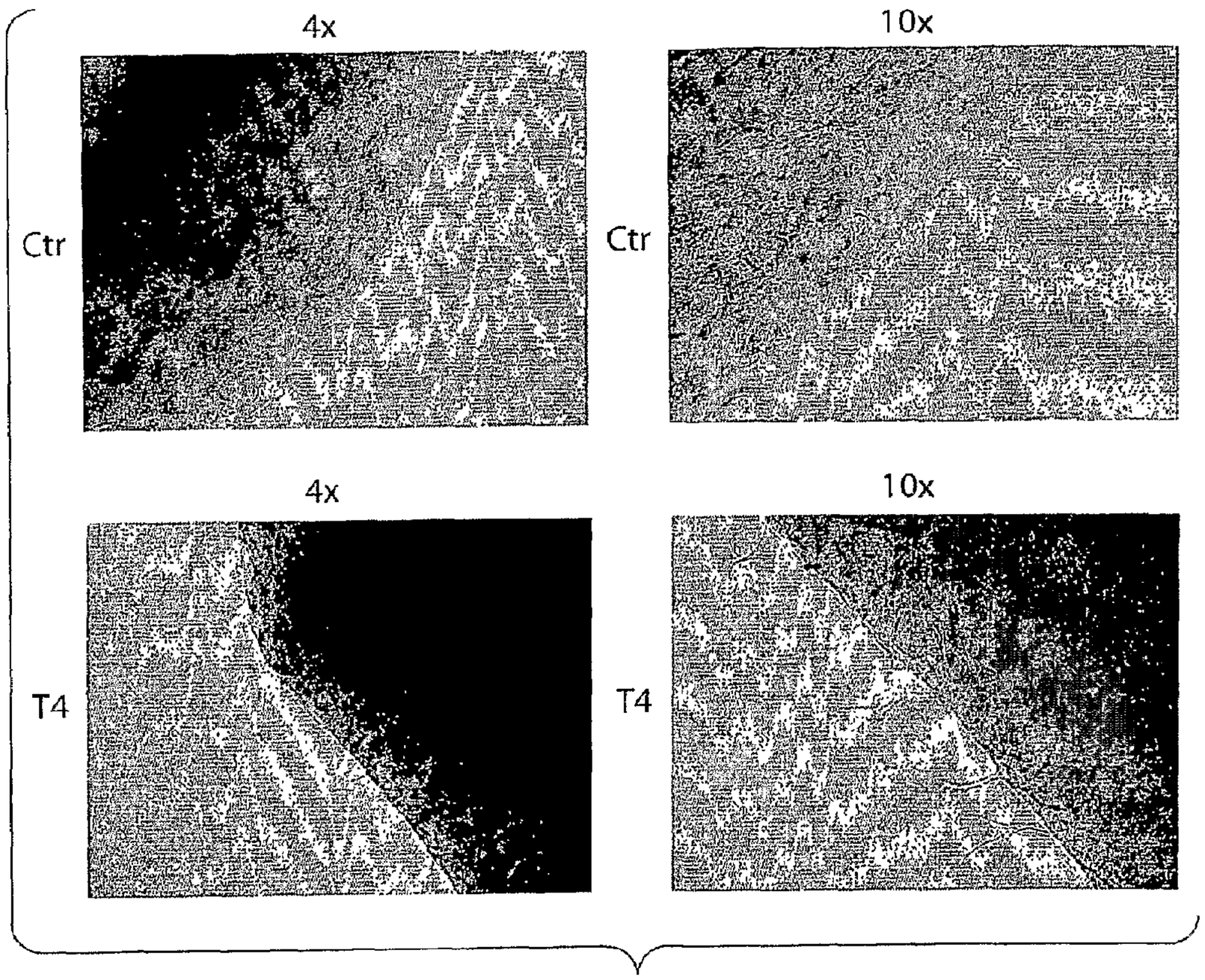


Fig. 9

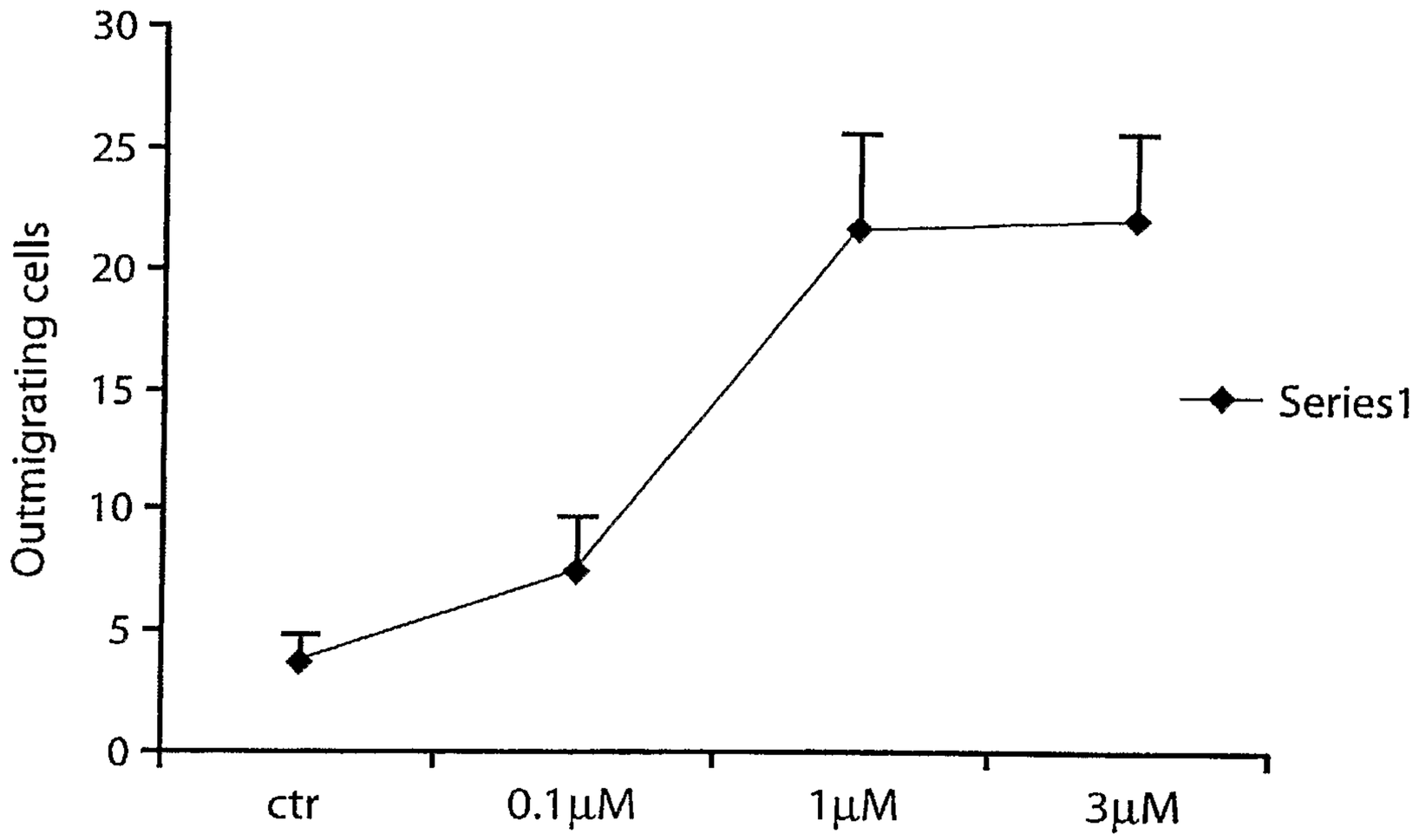


Fig. 10

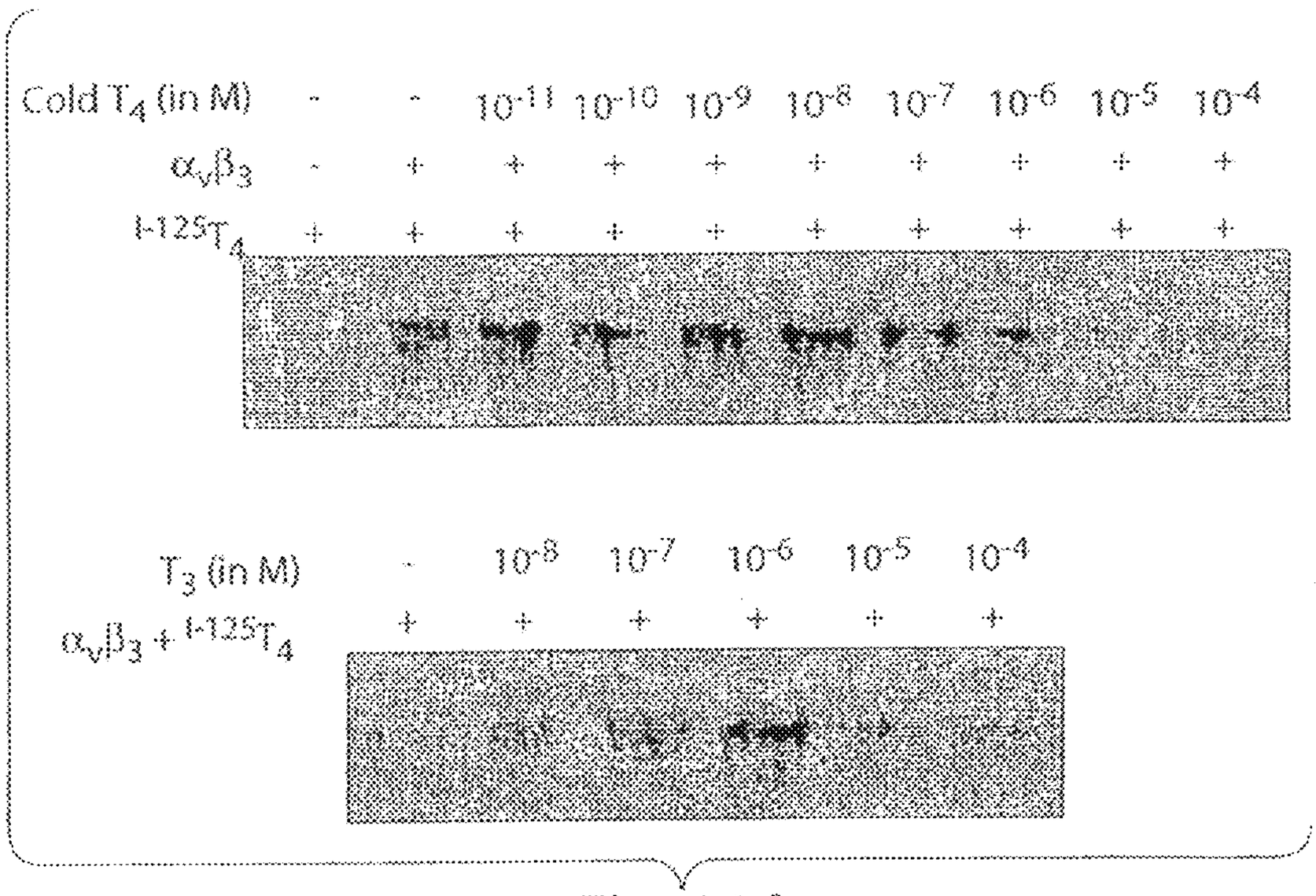


Fig. 11A

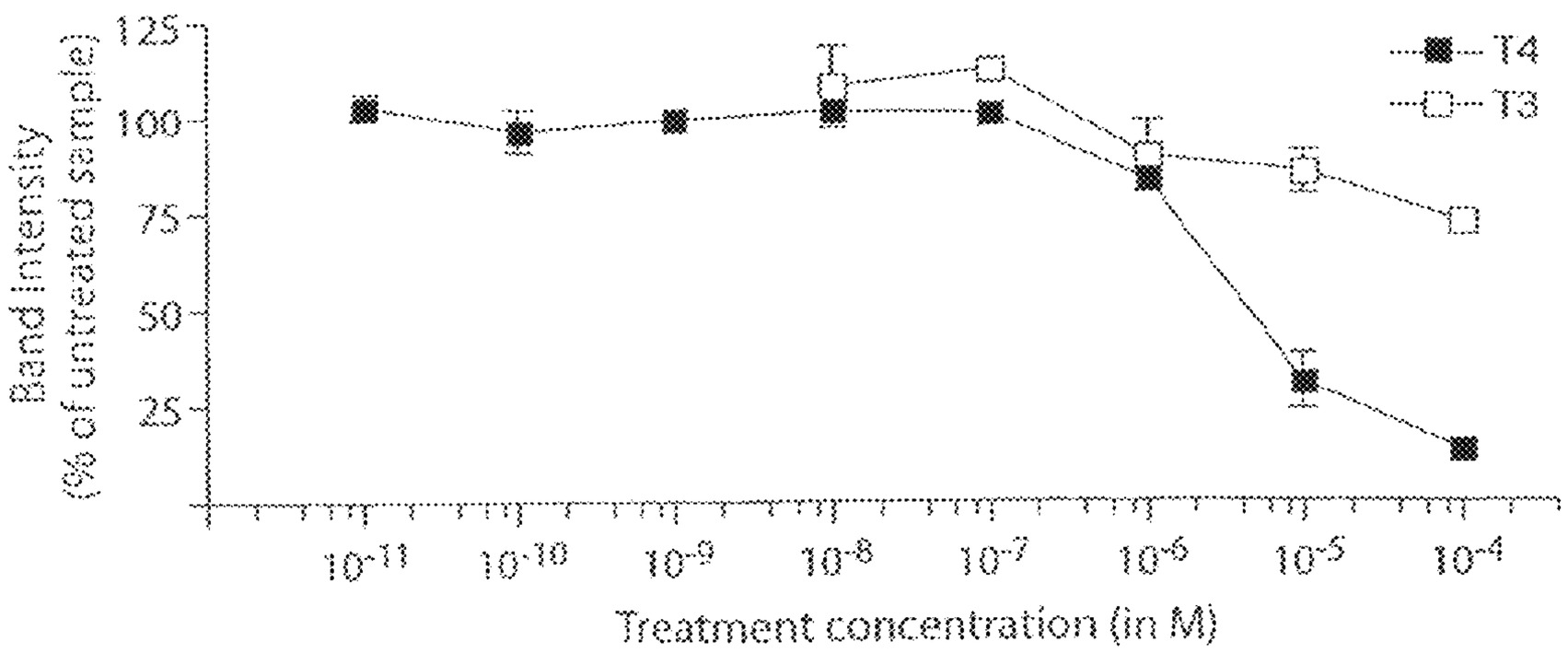


Fig. 11B

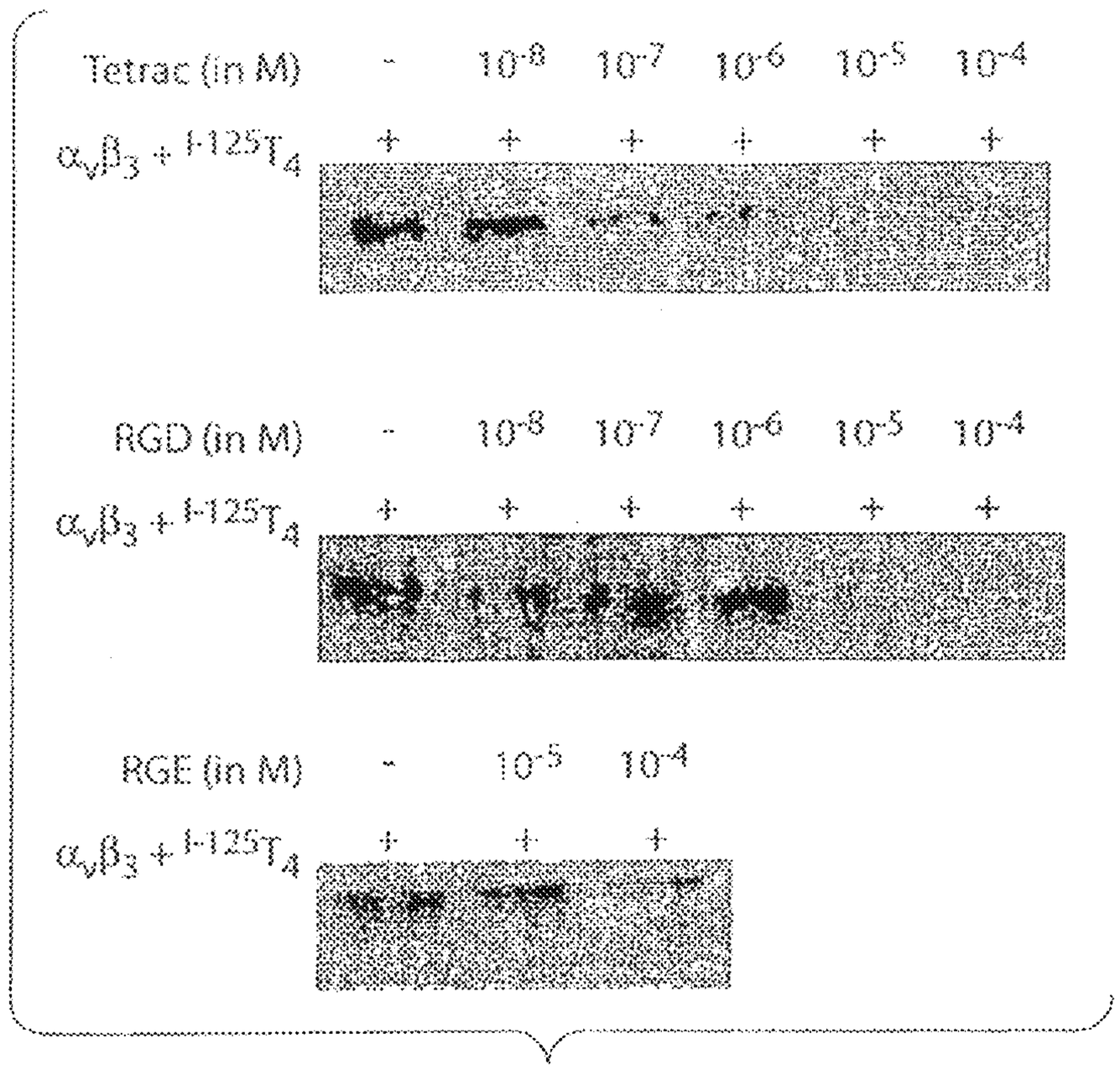


Fig. 12A

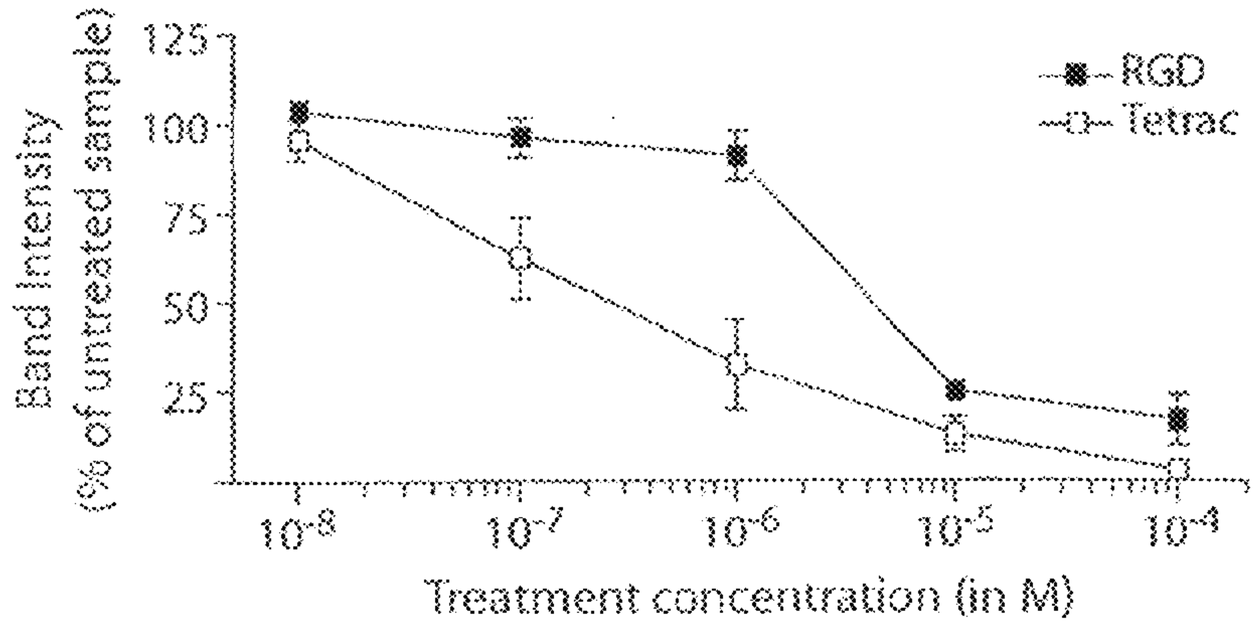


Fig. 12B

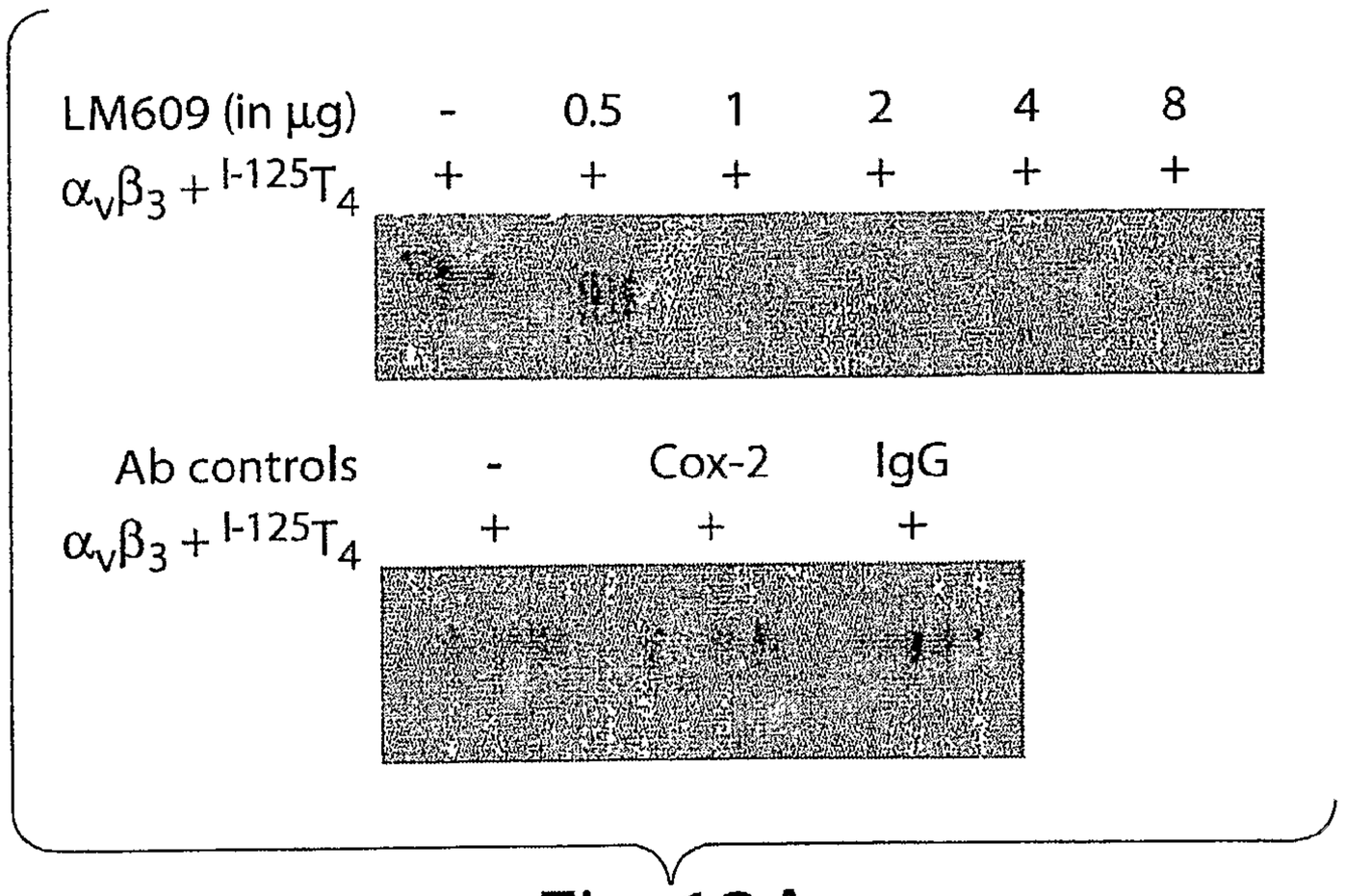


Fig. 13A

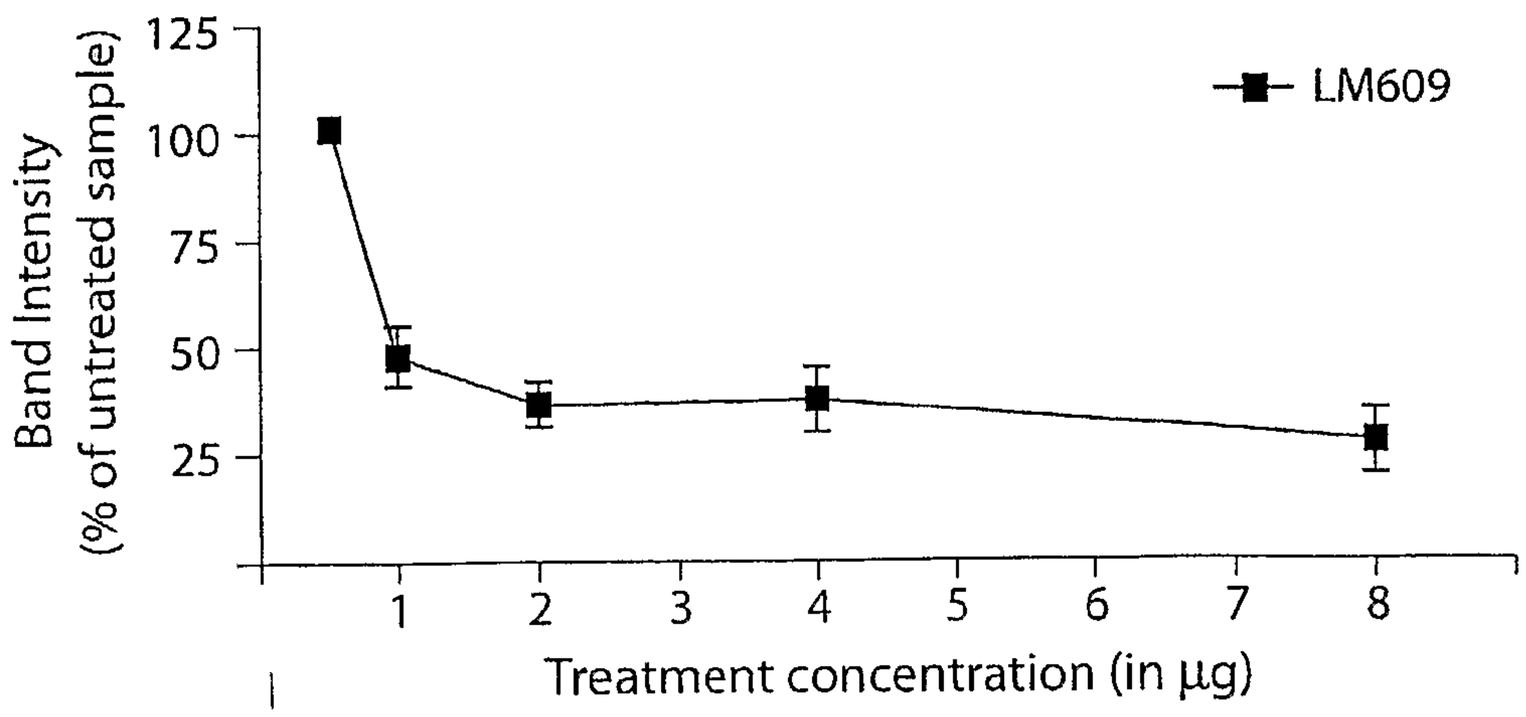


Fig. 13B

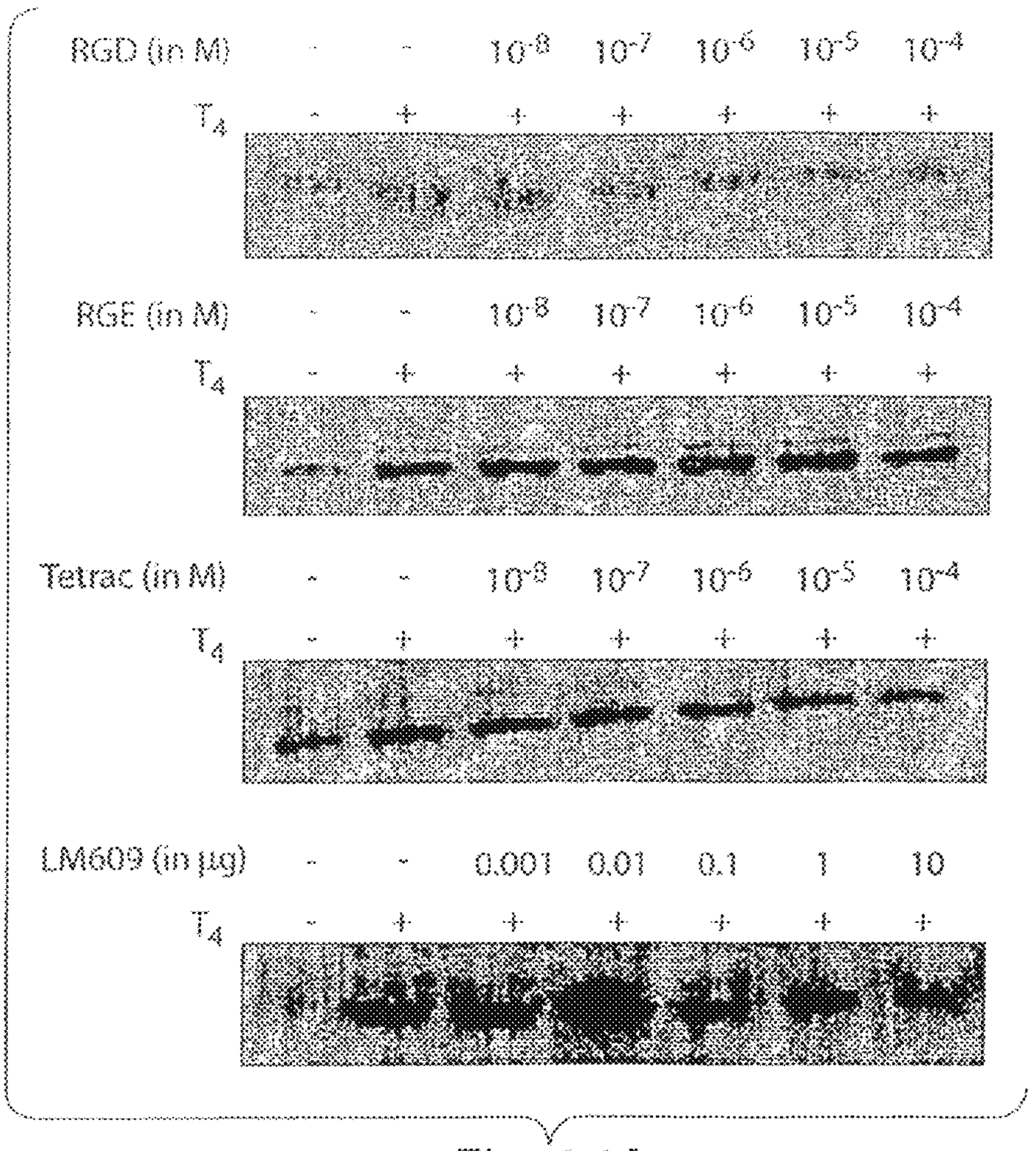


Fig. 14A

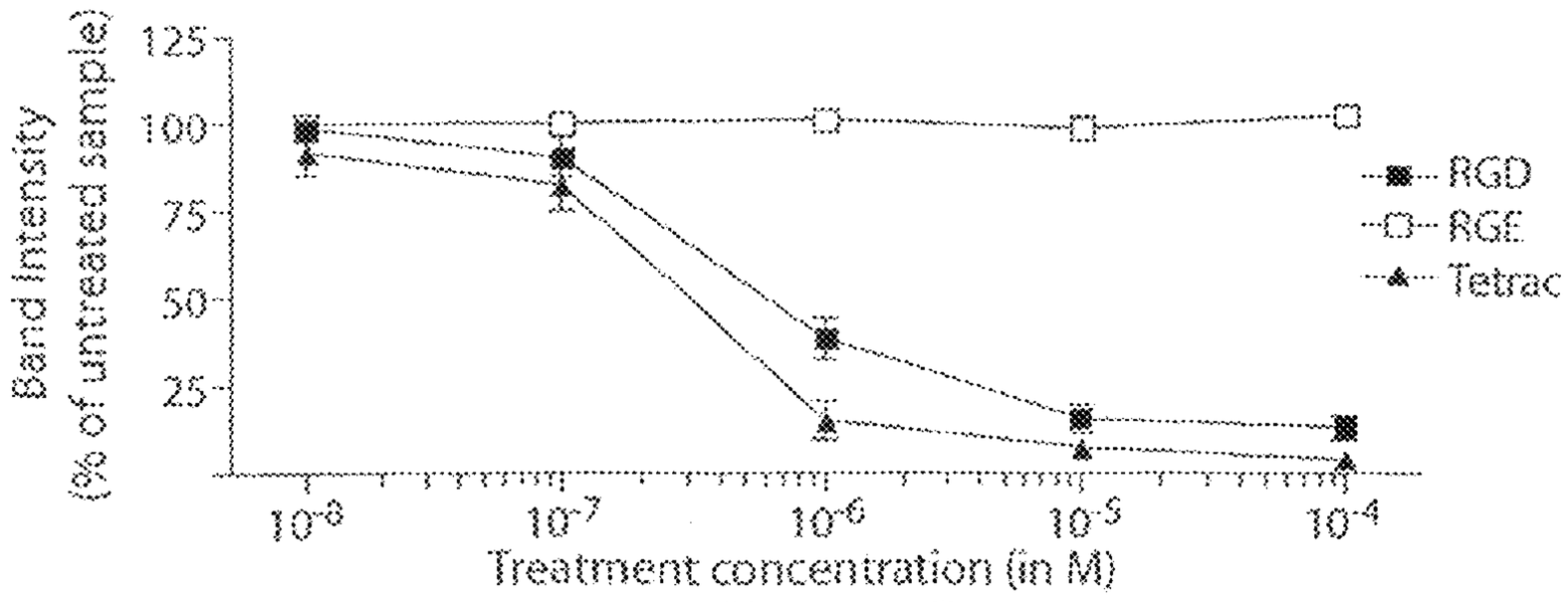


Fig. 14B

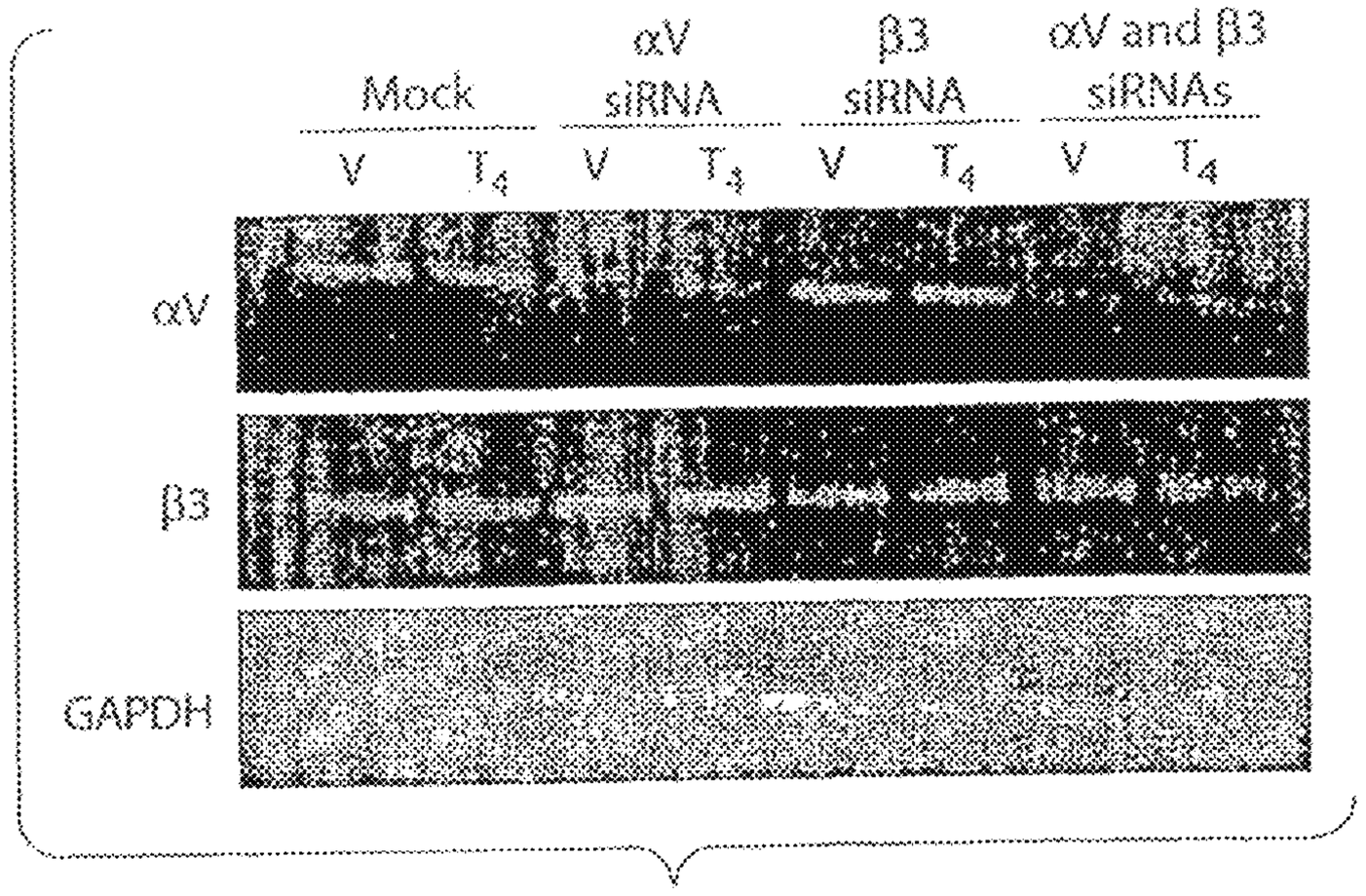


Fig. 15A

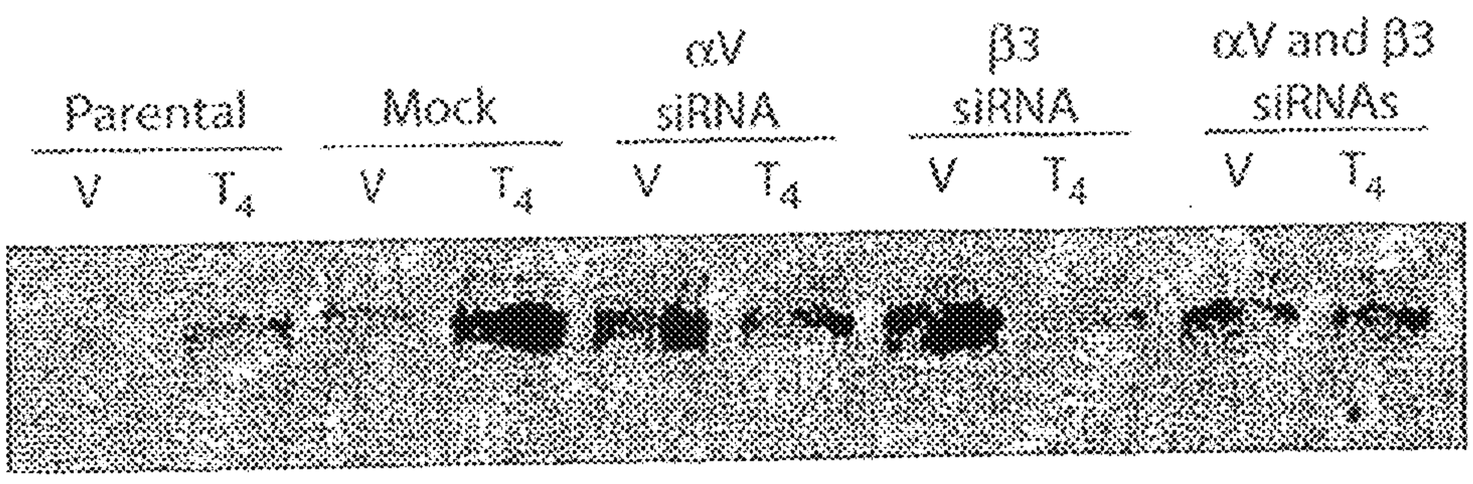


Fig. 15B

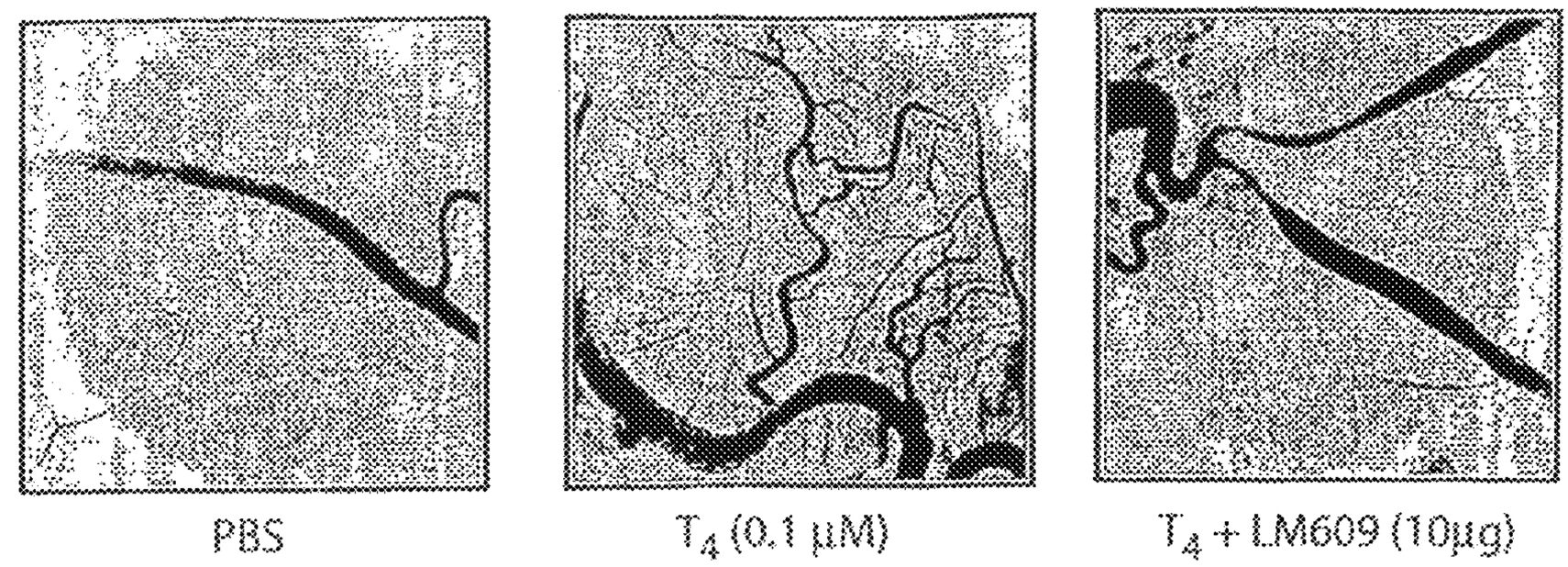


Fig. 16A

CAM treatment	# of Branches SEM	% Inhibition SEM
PBS	73 8	
T_4 (0.1 μM)	170 16	0
T_4 + LM609 (10 μg)	109 9	64 9

Fig. 16B

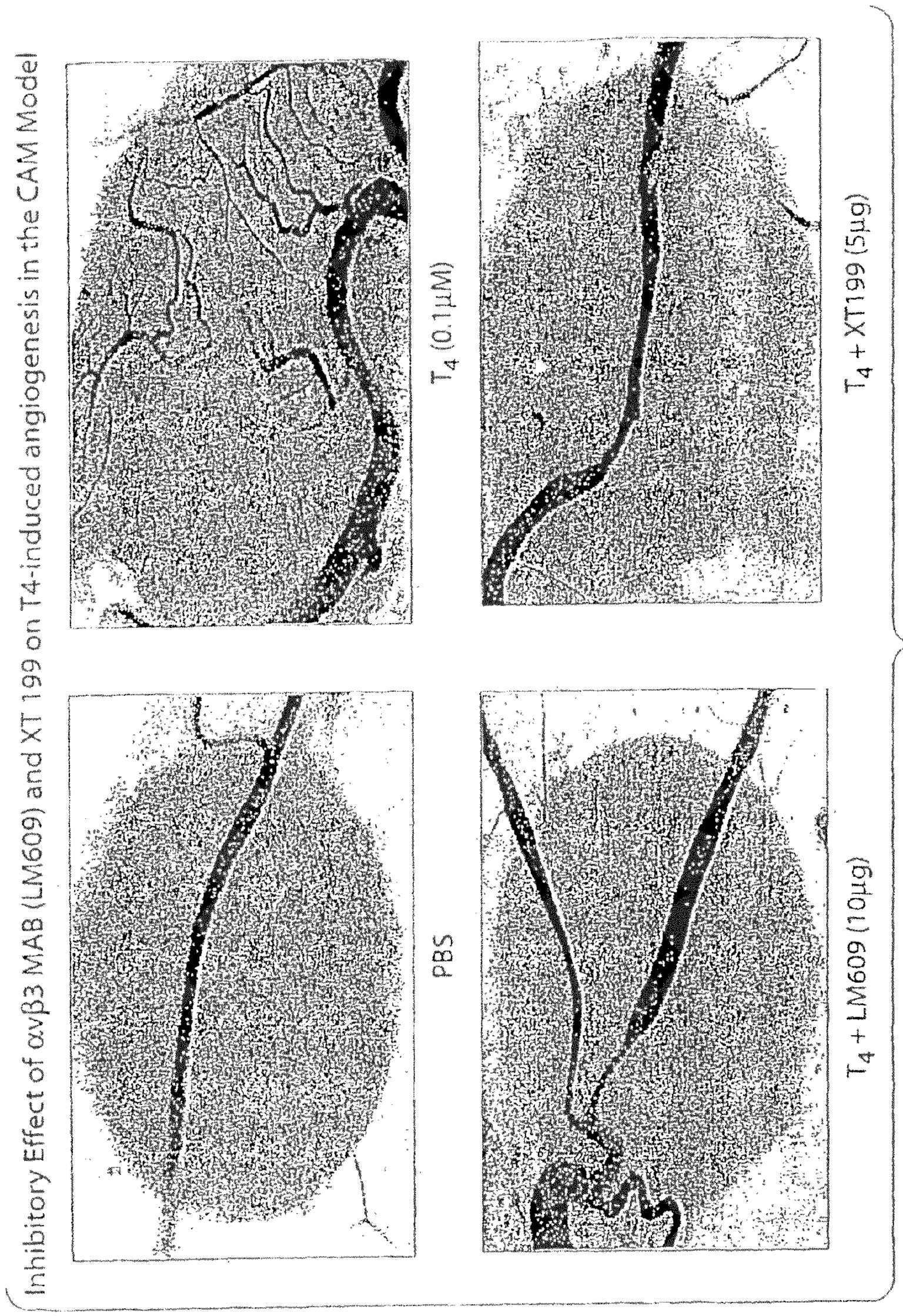

Inhibitory Effect of αvβ3 MAB (LM609) and XT 199 on T4-induced angiogenesis in the CAM Model
PBS
T4 (0.1μM)
T4 + LM609 (10μg)
T4 + XT199 (5μg)

Fig. 16C

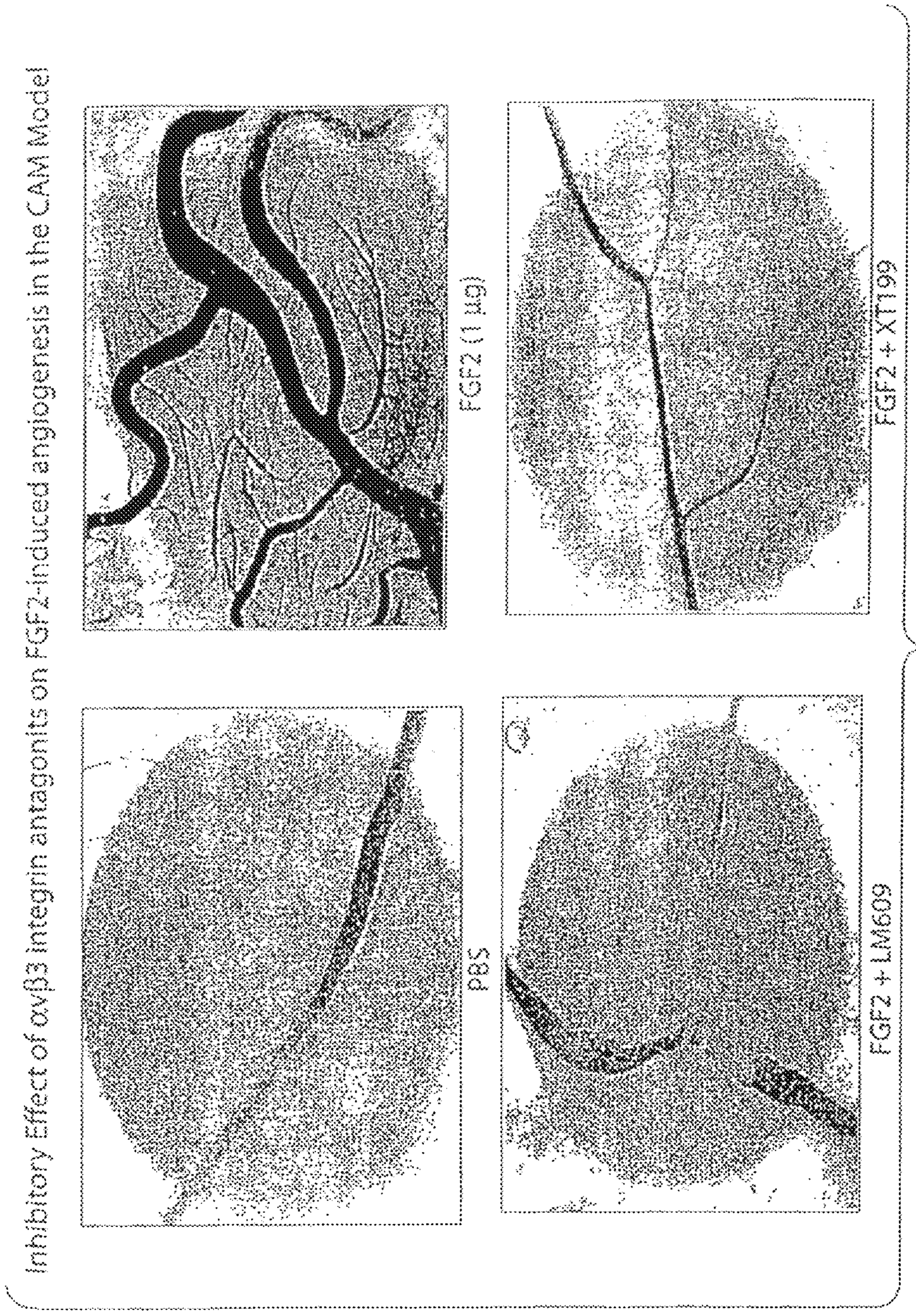

Inhibitory Effect of αvβ3 integrin antagonits on FGF2-induced angiogenesis in the CAM Model
PBS
FGF2 (1 μg)
FGF2 + LM609
FGF2 + XT199

Fig. 16D

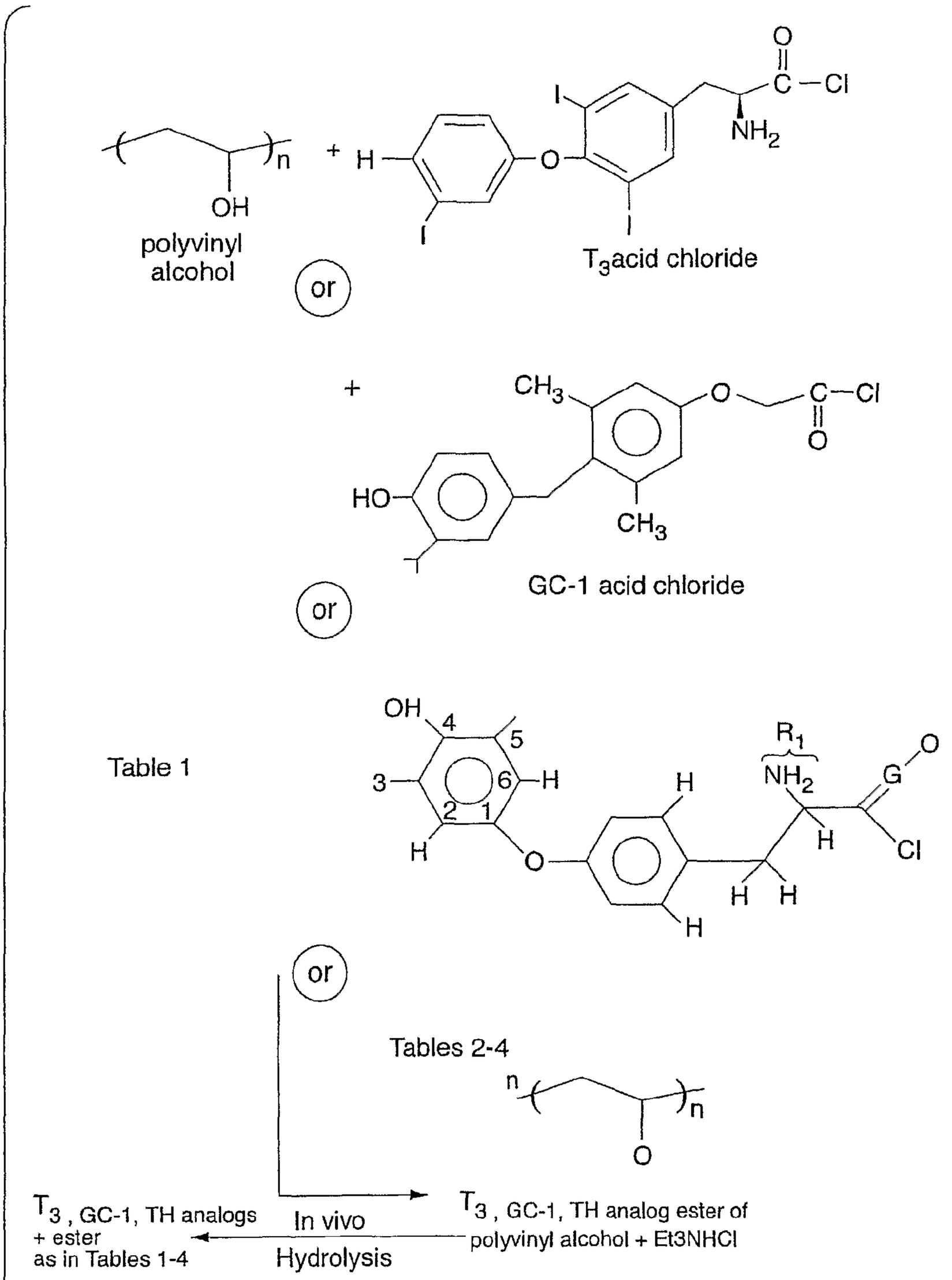

polyvinyl alcohol
or
T_3acid chloride
or
GC-1 acid chloride
or
Table 1
Tables 2-4
T_3 , GC-1, TH analogs + ester as in Tables 1-4
In vivo Hydrolysis
T_3 , GC-1, TH analog ester of polyvinyl alcohol + Et3NHCl

Fig. 17

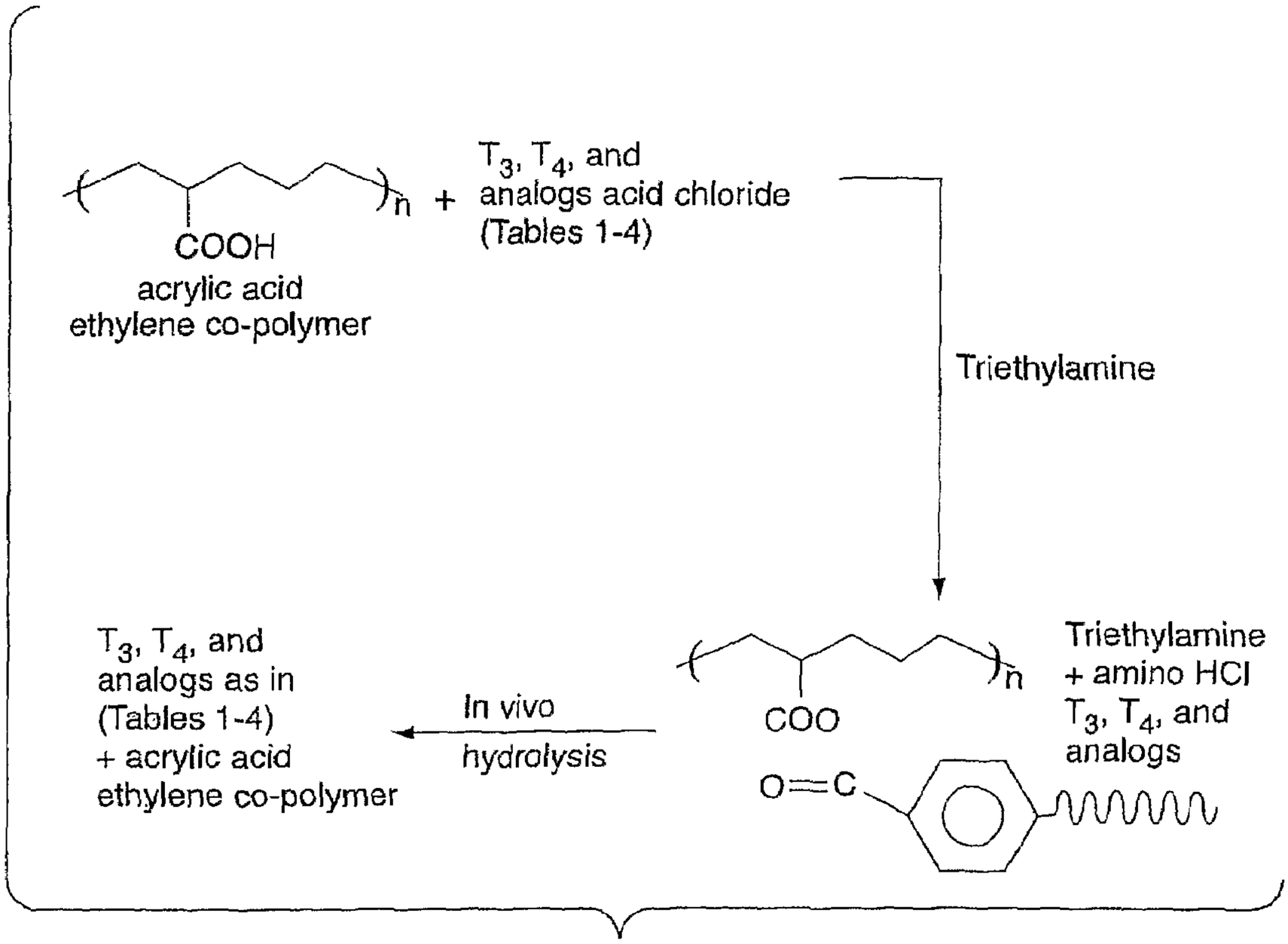

COOH
acrylic acid
ethylene co-polymer
n
+
T3, T4, and
analogs acid chloride
(Tables 1-4)
Triethylamine
COO
n
Triethylamine
+ amino HCl
T3, T4, and
analogs
O=C
In vivo
hydrolysis
T3, T4, and
analogs as in
(Tables 1-4)
+ acrylic acid
ethylene co-polymer

Fig. 18

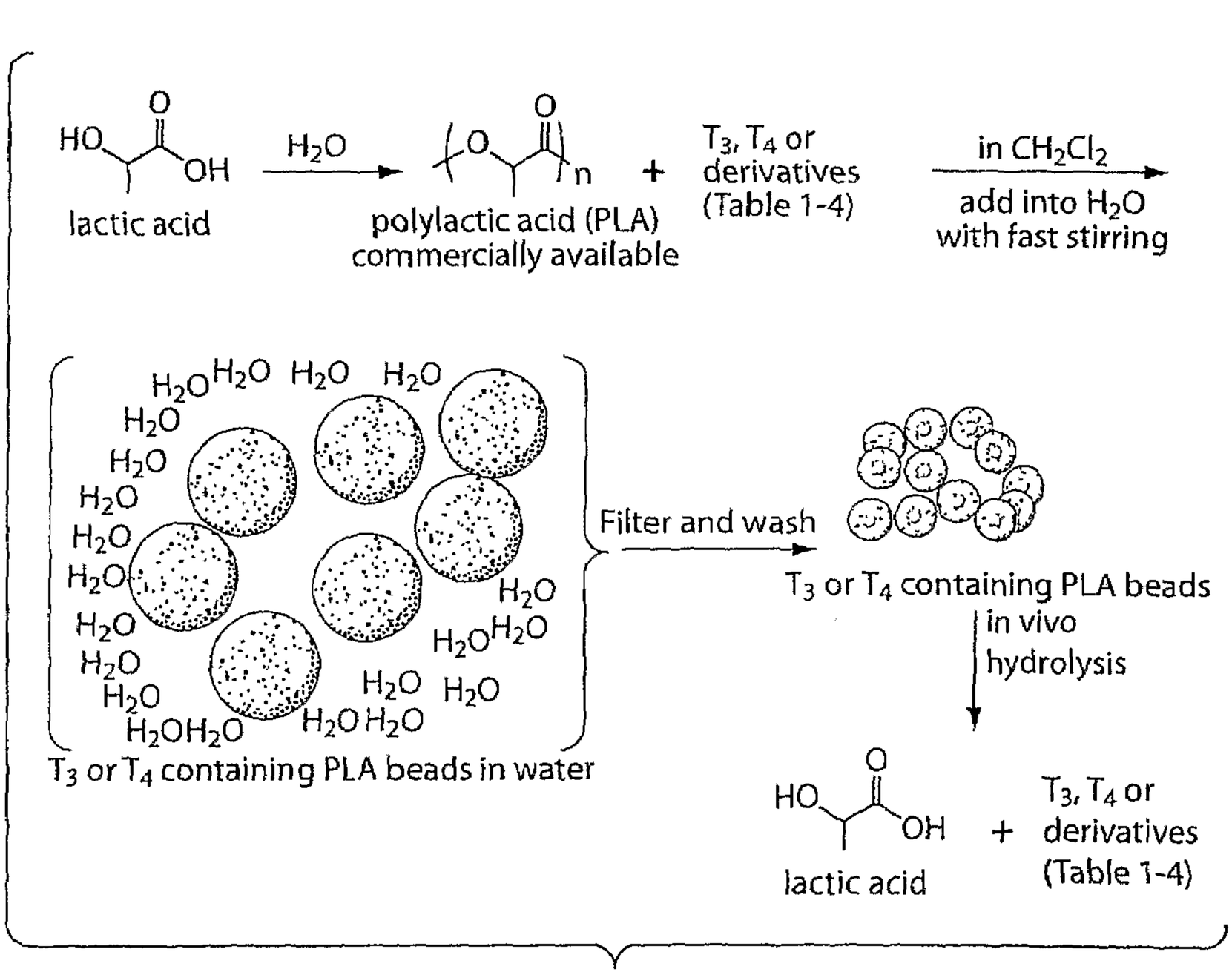
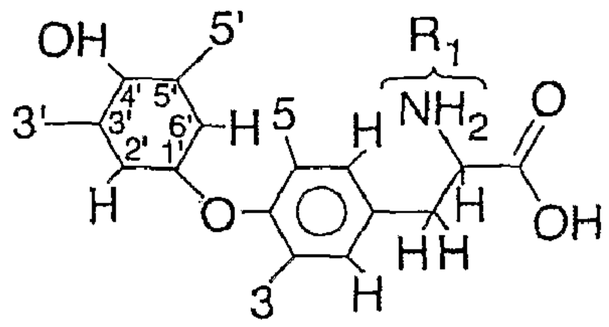

lactic acid
H_2O
polylactic acid (PLA)
commercially available
T_3, T_4 or
derivatives
(Table 1-4)
in CH_2Cl_2
add into H_2O
with fast stirring
T_3 or T_4 containing PLA beads in water
Filter and wash
T_3 or T_4 containing PLA beads
in vivo
hydrolysis
lactic acid
T_3, T_4 or
derivatives
(Table 1-4)

Fig. 19

Table A

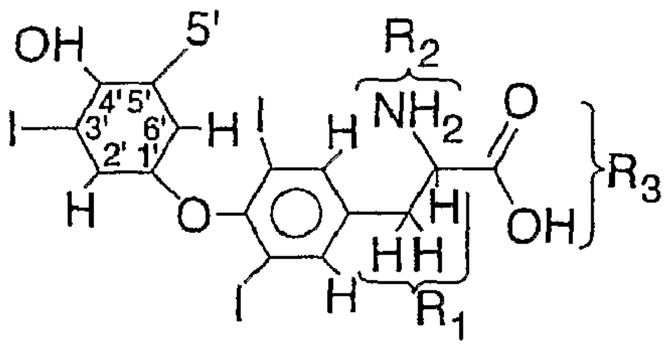


3'	5'	3	5	R_1	Analogue
I	I	I	I	NH_2	L-T_4
I	H	I	I	NH_2	L-T_3
I	I	I	H	NH_2	rT_3
H	H	I	I	NH_2	3,5-L-T_2
I	I	H	H	NH_2	3',5'-L-T_2
I	H	I	H	NH_2	3,3'-L-T_2
I	H	H	H	NH_2	3'-L-T_3
Br	Br	Br	Br	NH_2	3,5,3'-tetra-bromo-L-thyronine
H	H	Br	Br	NH_2	3,5,3',-dibromo-L-thyronine
Isop[a]	H	Me[b]	Me	NH_2	*DIMIT*
Isop	H	Me	Me	NH-$COCH_3$	*N*-acetyl *DIMIT*

[a] Isop, isopropyl
[b] Me, methyl

Fig. 20A

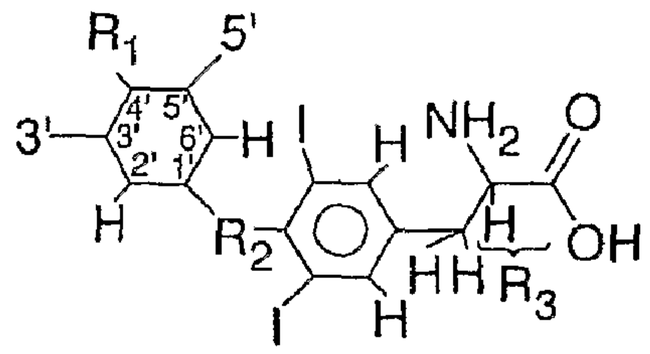
Table B



R_1	R_2	R_3	5'	Analogue
CH_2CH	H	CO_2H	I	3,5,3',5'-tetraiodo-thyropropionic acid
CH_2	H	CO_2H	I	3,5,3',5'-tetraiodo-thyroacetic acid
CH_2	H	CO_2H	H	3,5,3'-triiodothyroa-cetic acid
CH_2CH	NH_2	COC_2H_5	I	L-T_4 ethylester
CH_2CH	NH_2	H	H	3,5,3'-triiodothyrona-mine

Fig. 20B

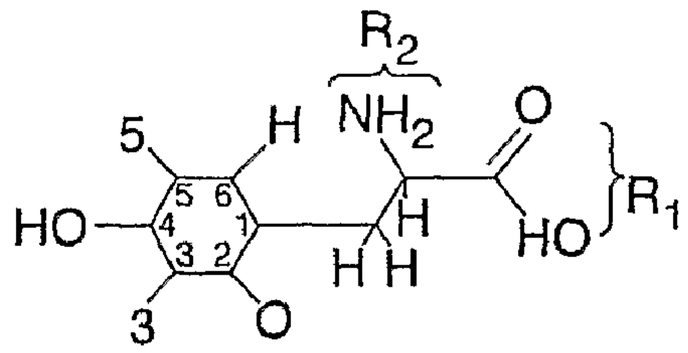
Table C



R_1	R_2	R_3	3'	5'	3	5	Analogue
H	O	L	H	H	I	I	4' -deoxy T_2
OH	S	L	I	H	I	I	S-bridged T_3
OH	O	D	I	I	I	I	D-T_4
OH	O	D	I	H	I	I	D-T_3

Fig. 20C

Table D



3	5	R_1	R_3	Analogue
I	I	COOH	NH_2	3,5-diiodo-L-tyrosine
Br	Br	COOH	NH_2	3,5-dibromo-L-tyrosine
Me	Me	COOH	NH_2	3,5-dimethyl-DL-tyrosine
NO_2	NO_2	COOH	NH_2	3,5-dinitro-L-tyrosine
I	H	COOH	NH_2	3-iodo-L-tyrosine
NO_2	H	COOH	NH_2	3-nitro-L-tyrosine
H	H	COOH	NH_2	L-tyrosine
I	I	H	NH_2	3,5-diiodotyramine
H	H	H	NH_2	tyramine
I	I	COOH	H	3-(3,5-diiodo-4-hydroxy-phenyl) propionic acid
H	H	COOH	H	3-(p-hydroxy-phenyl) propionic acid

Fig. 20D

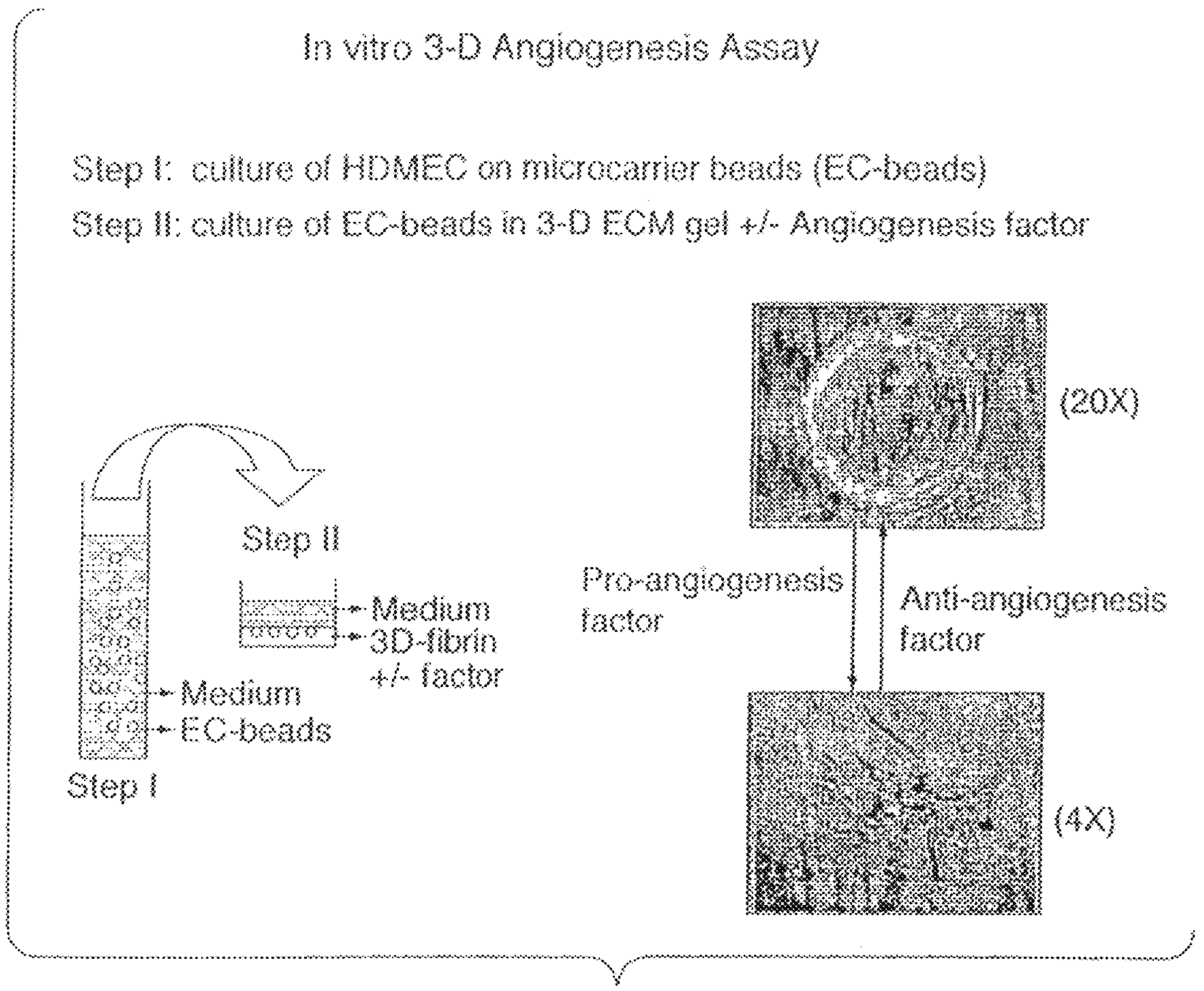

In vitro 3-D Angiogenesis Assay
Step I: culture of HDMEC on microcarrier beads (EC-beads)
Step II: culture of EC-beads in 3-D ECM gel +/- Angiogenesis factor
Step II
Medium
3D-fibrin
+/- factor
Medium
EC-beads
Step I
(20X)
Pro-angiogenesis factor
Anti-angiogenesis factor
(4X)

Fig. 21

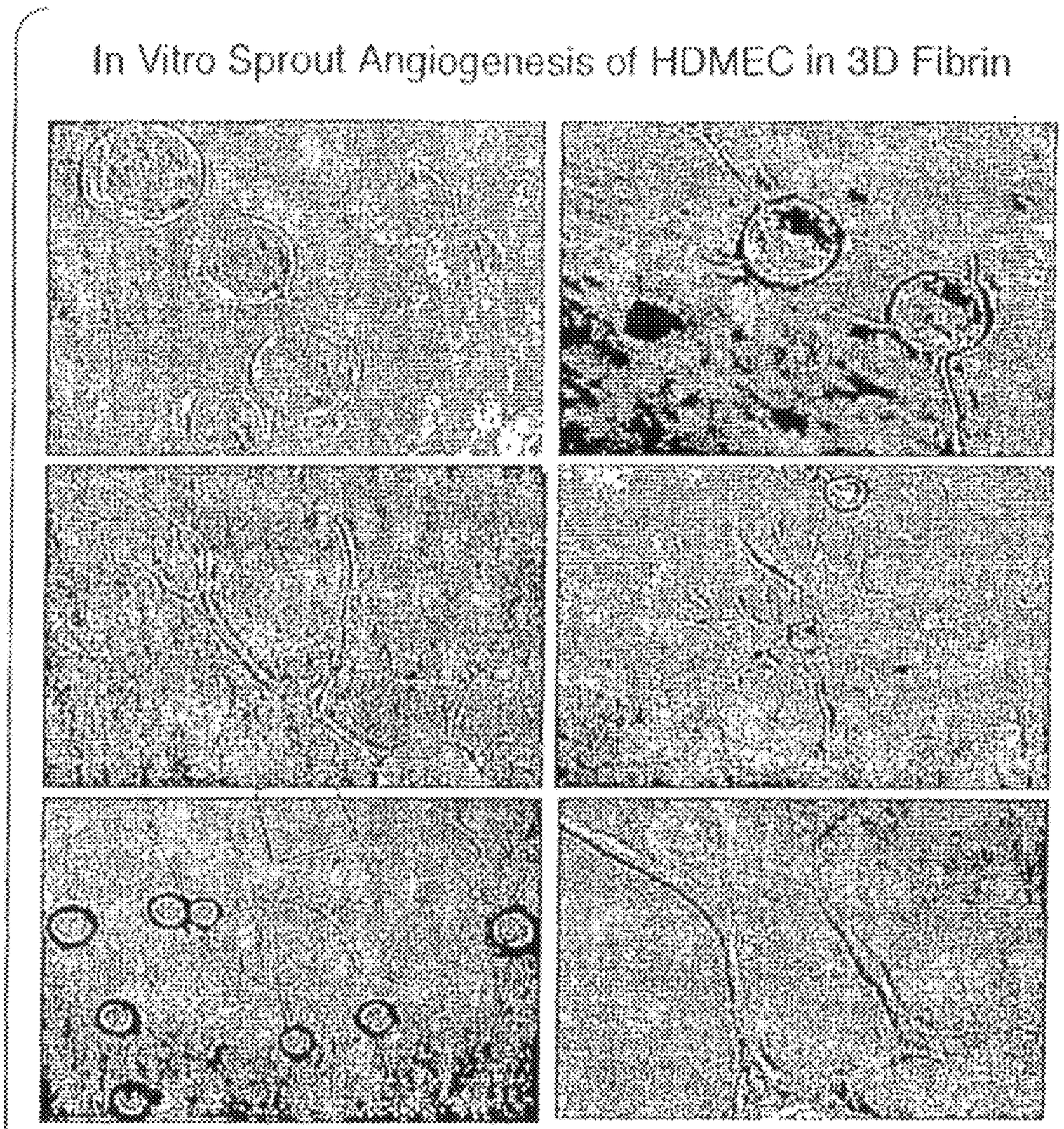


Fig. 22

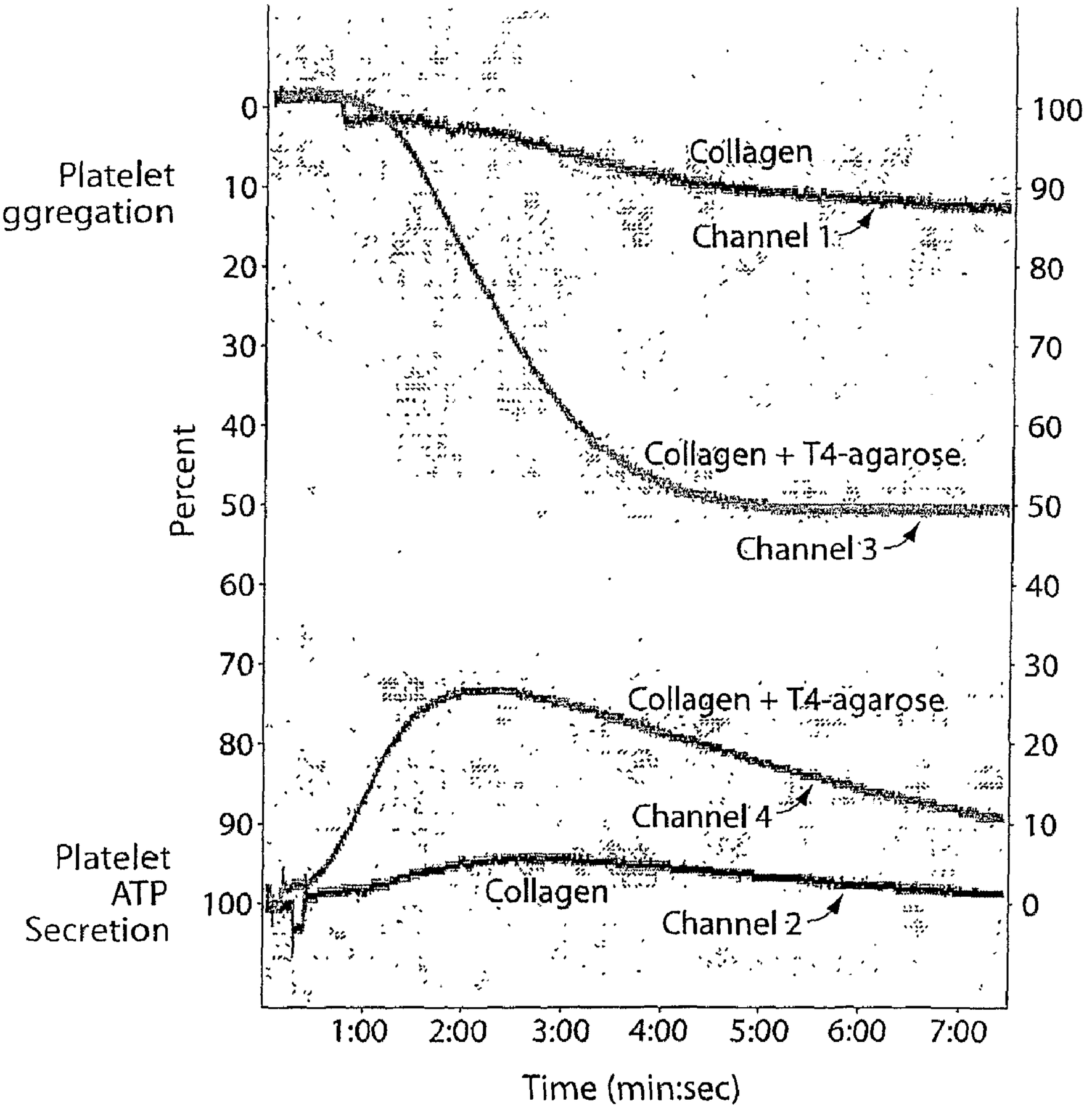

Platelet aggregation
Percent
0
10
20
30
40
50
60
70
80
90
100
Collagen
Channel 1
Collagen + T4-agarose
Channel 3
Collagen + T4-agarose
Channel 4
Platelet ATP Secretion
100
90
80
70
60
50
40
30
20
10
0
Collagen
Channel 2
1:00
2:00
3:00
4:00
5:00
6:00
7:00
Time (min:sec)

Fig. 23A

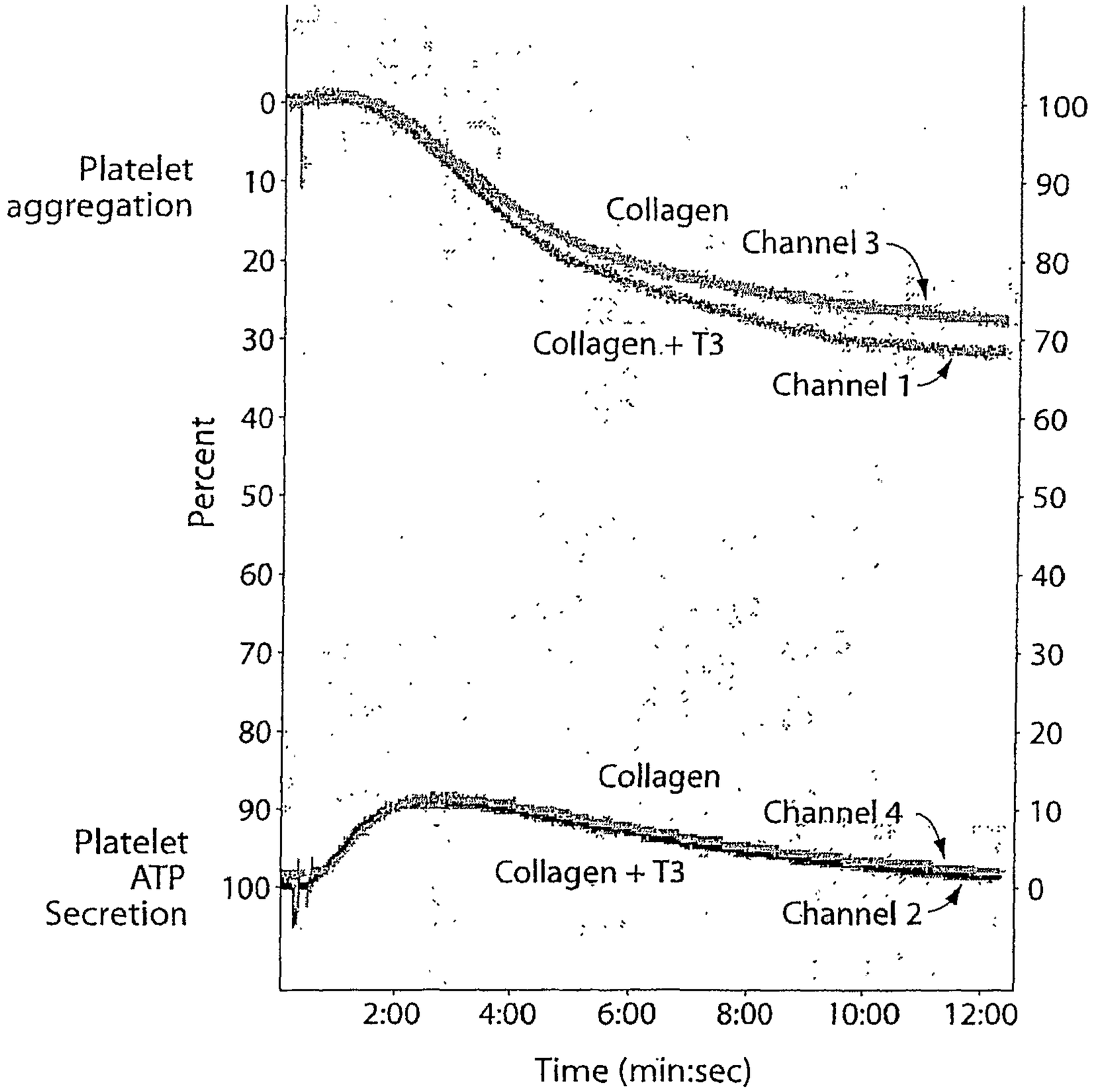

Platelet aggregation
Collagen
Channel 3
Collagen + T3
Channel 1
Percent
Platelet ATP Secretion
Collagen
Channel 4
Collagen + T3
Channel 2
0
10
20
30
40
50
60
70
80
90
100
2:00
4:00
6:00
8:00
10:00
12:00
Time (min:sec)

Fig. 23B

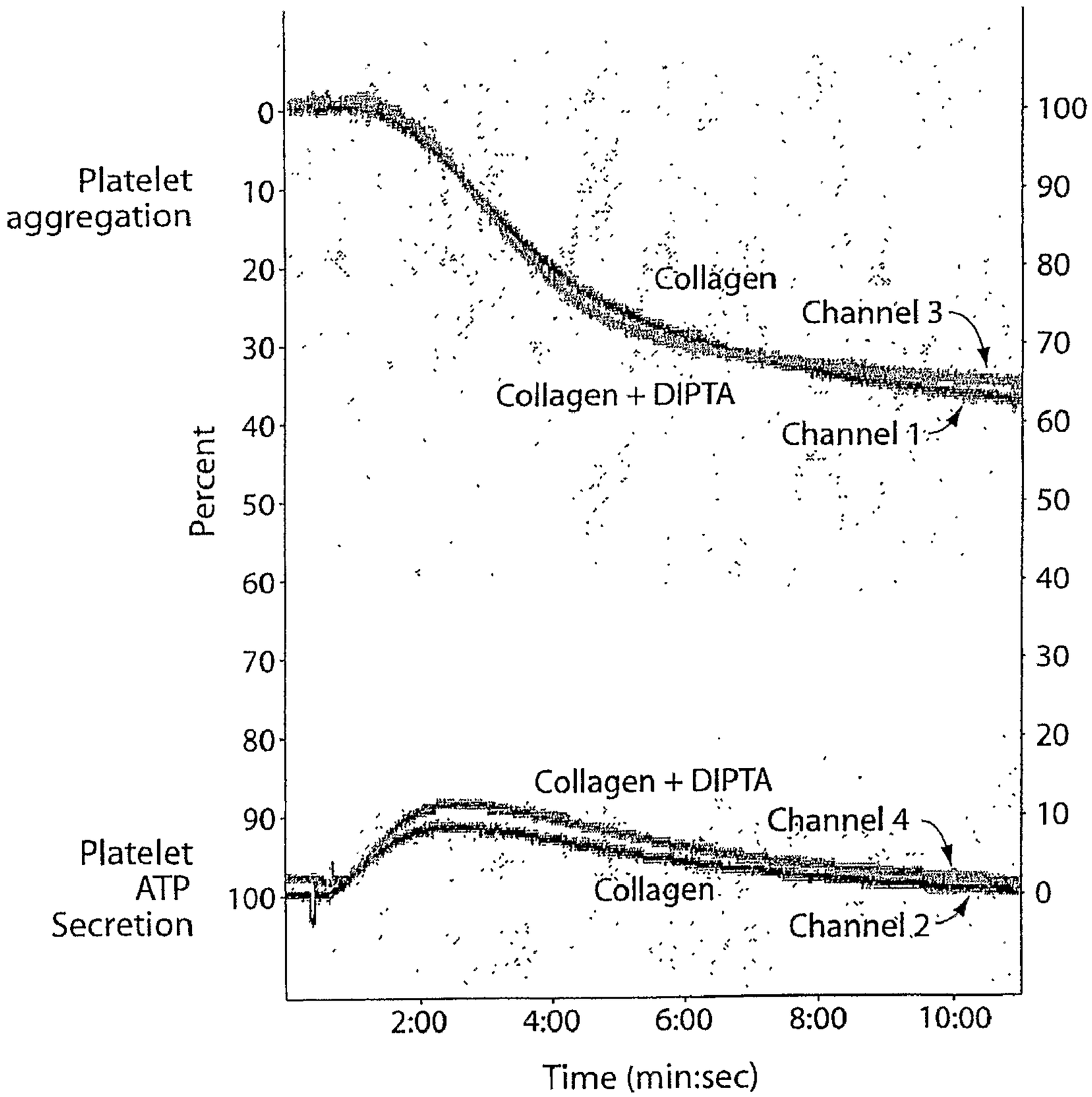

Platelet aggregation
Collagen
Channel 3
Collagen + DIPTA
Channel 1
Percent
Collagen + DIPTA
Channel 4
Platelet ATP Secretion
Collagen
Channel 2
0
10
20
30
40
50
60
70
80
90
100
2:00
4:00
6:00
8:00
10:00
Time (min:sec)

Fig. 23C

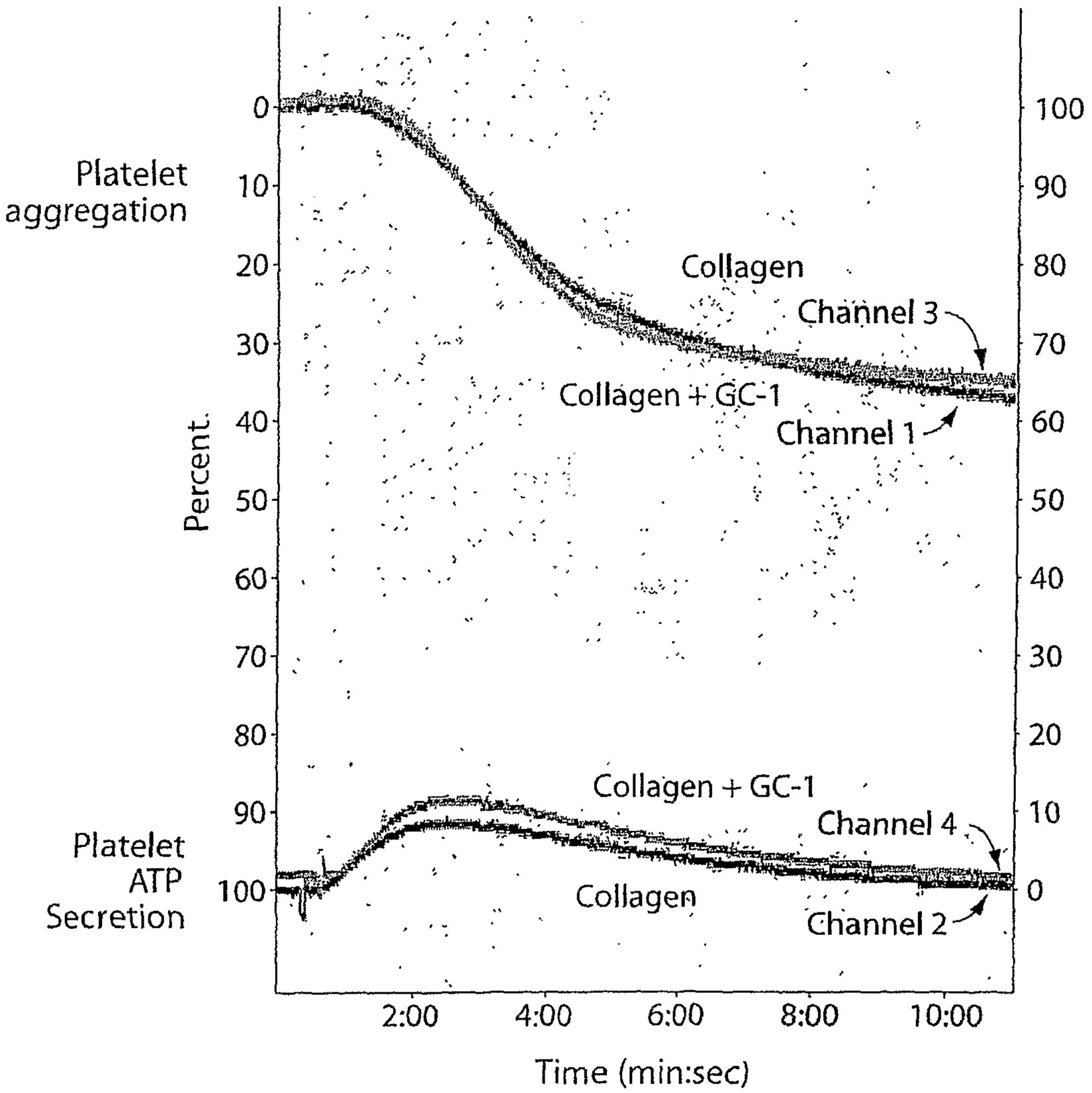

Platelet aggregation
Platelet ATP Secretion
Percent
0
10
20
30
40
50
60
70
80
90
100
Collagen
Channel 3
Collagen + GC-1
Channel 1
Collagen + GC-1
Channel 4
Collagen
Channel 2
2:00
4:00
6:00
8:00
10:00
Time (min:sec)

Fig. 23D

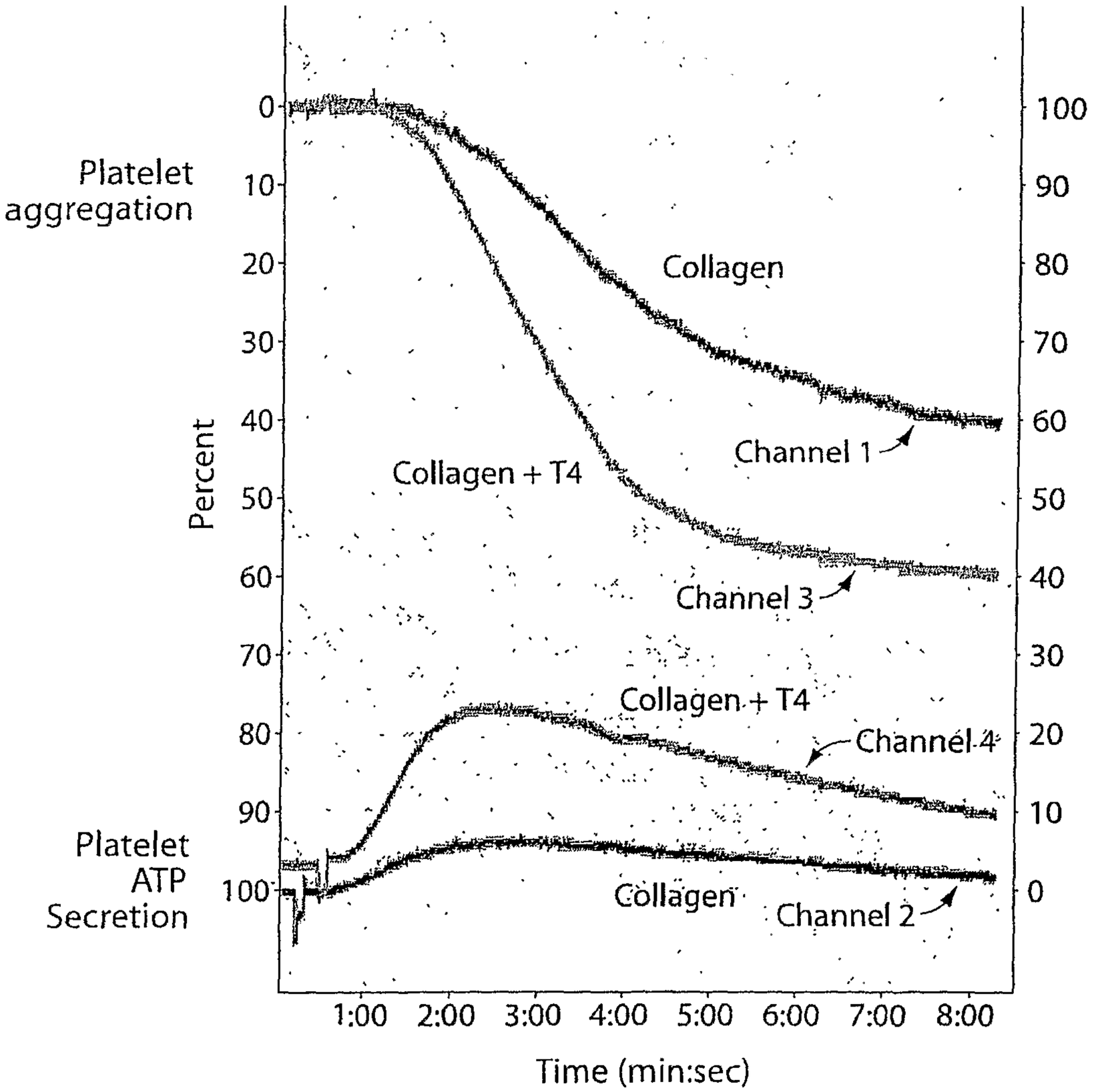

Platelet aggregation
Percent
0
10
20
30
40
50
60
70
80
90
100
Collagen
Channel 1
Collagen + T4
Channel 3
Collagen + T4
Channel 4
Platelet ATP Secretion
Collagen
Channel 2
100
90
80
70
60
50
40
30
20
10
0
1:00
2:00
3:00
4:00
5:00
6:00
7:00
8:00
Time (min:sec)

Fig. 23E

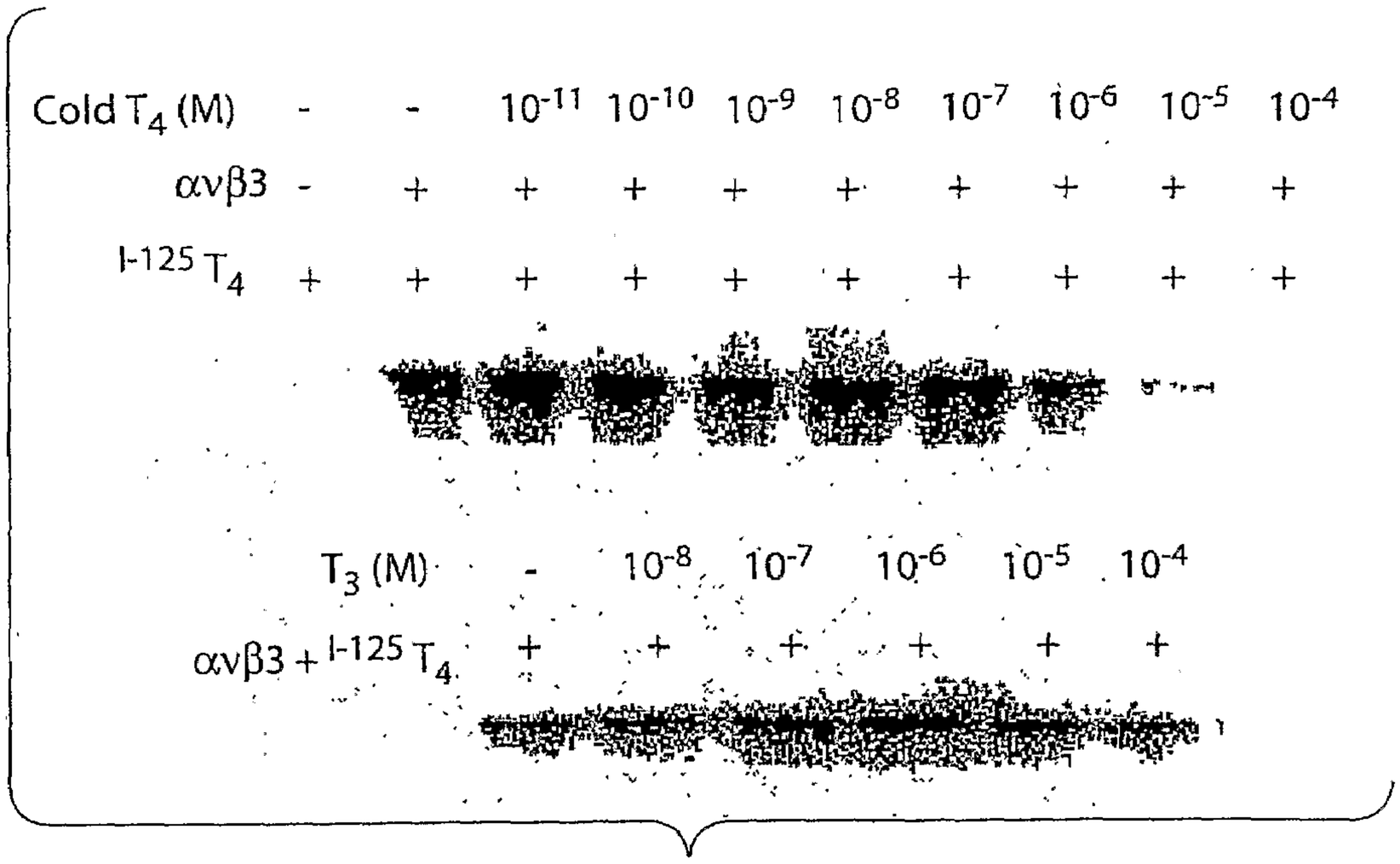


Fig. 24A

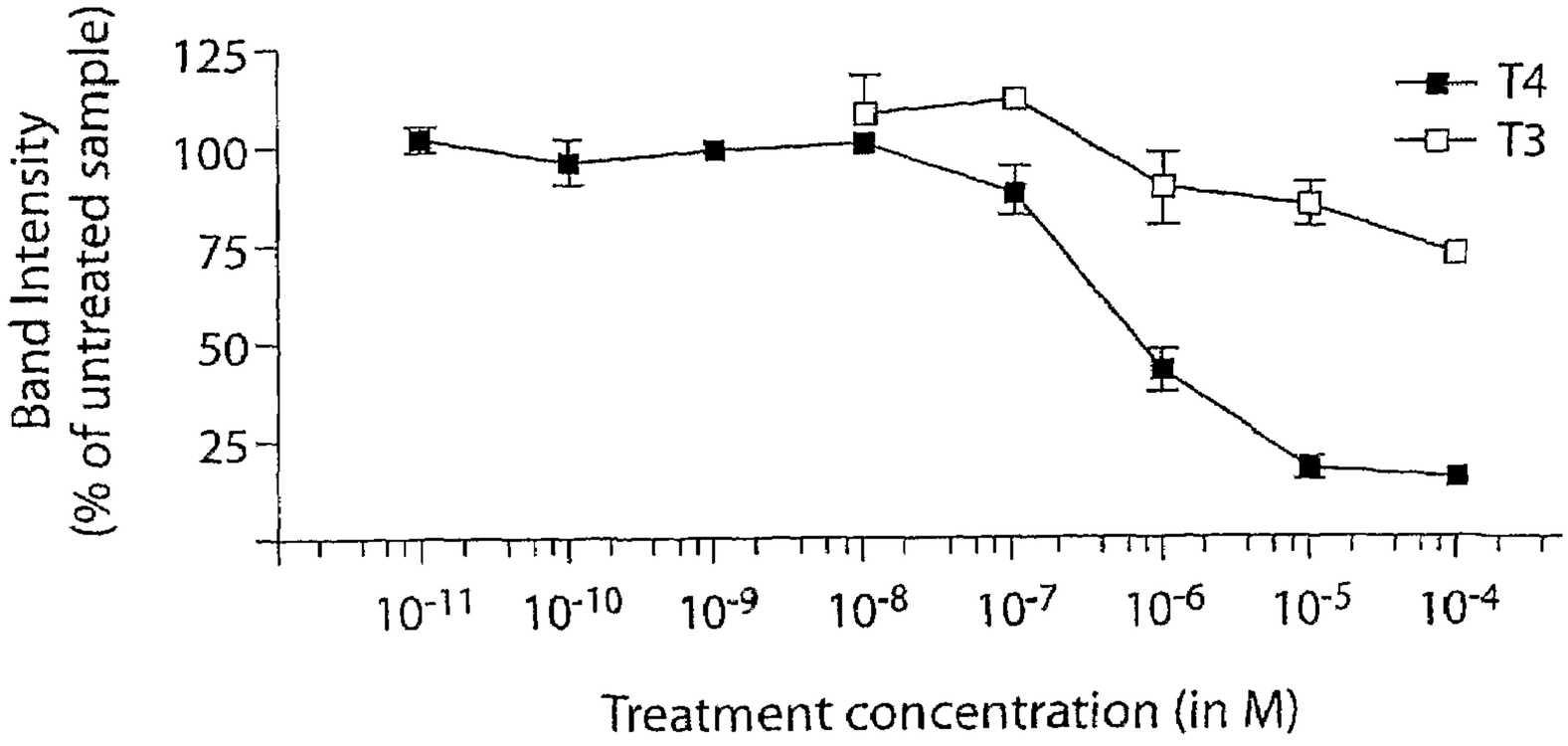


Fig. 24B

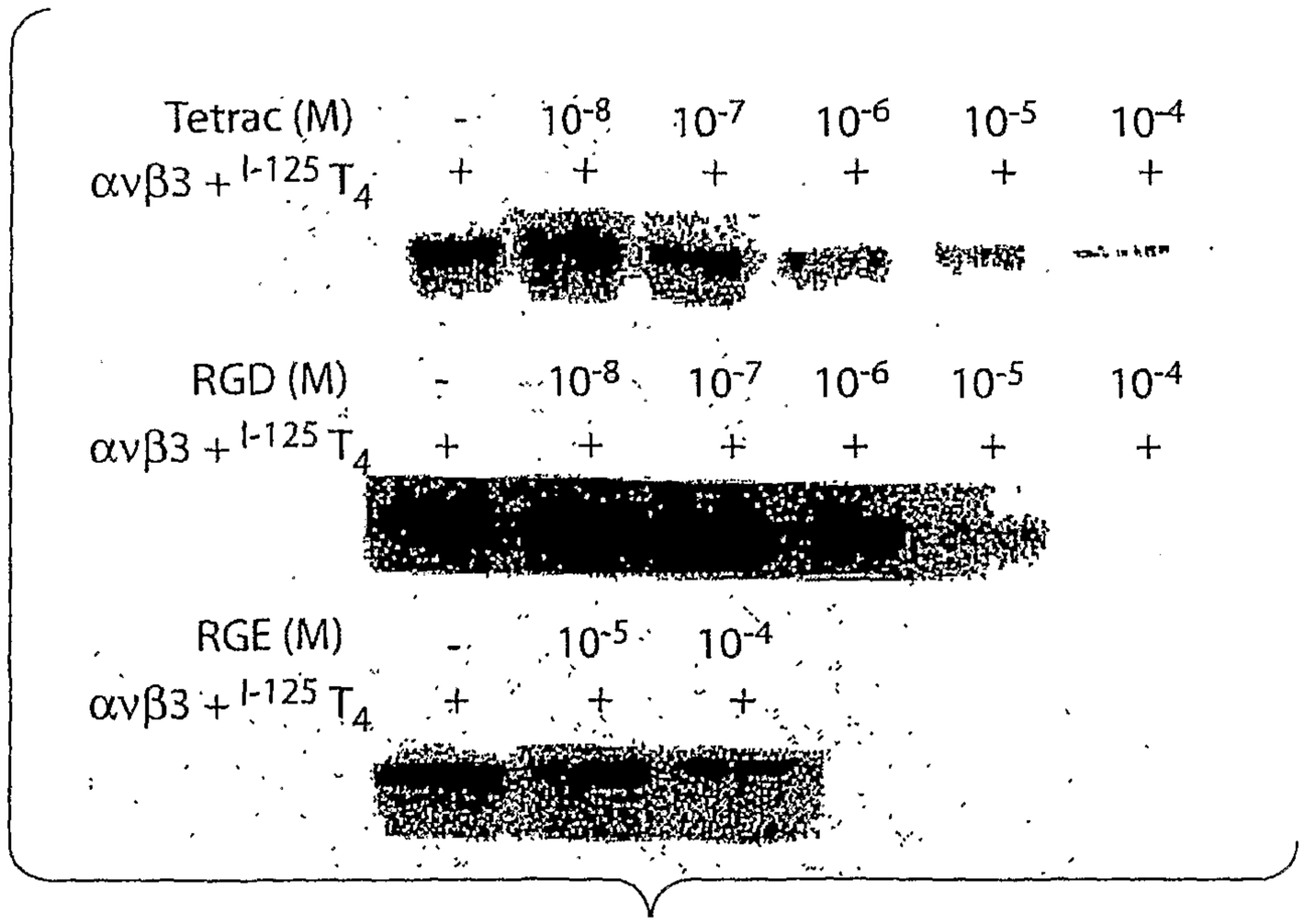


Fig. 25A

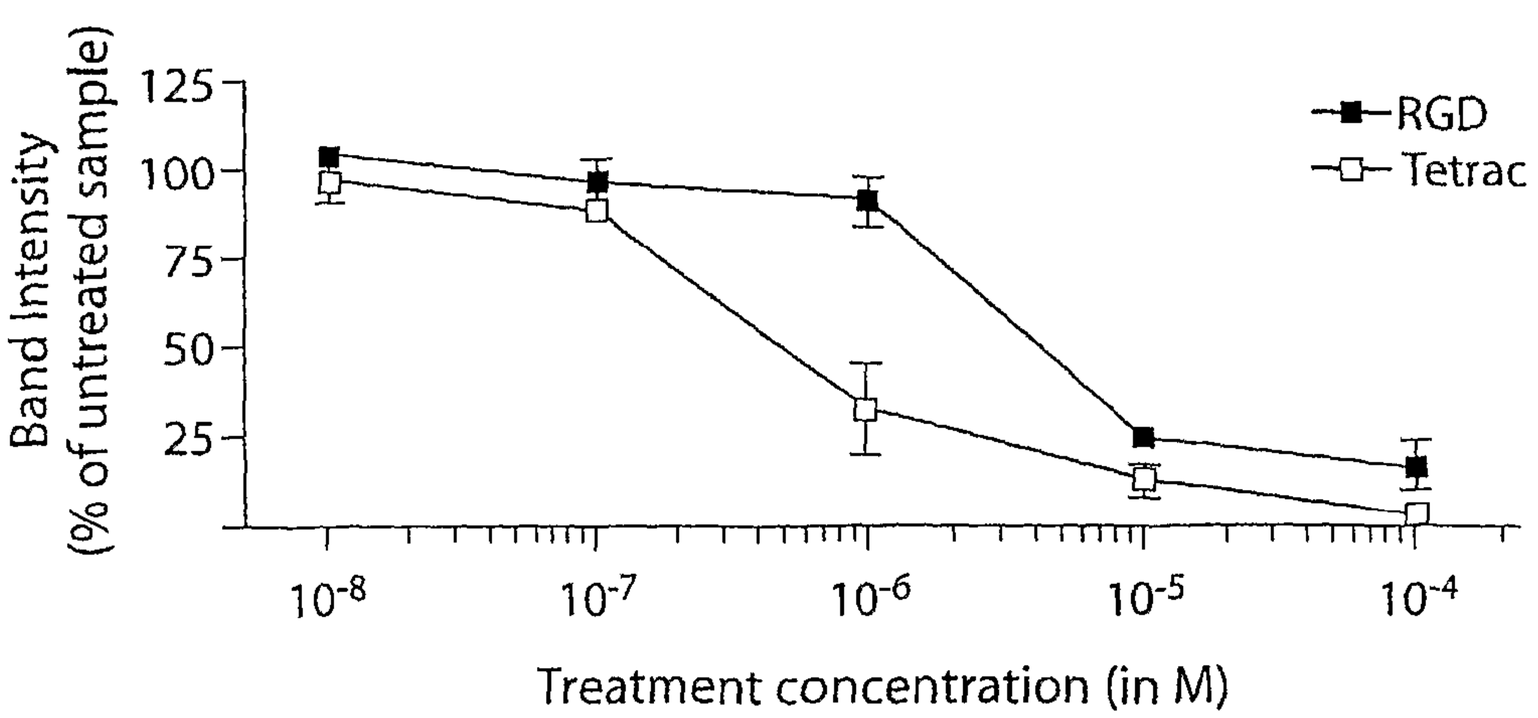


Fig. 25B

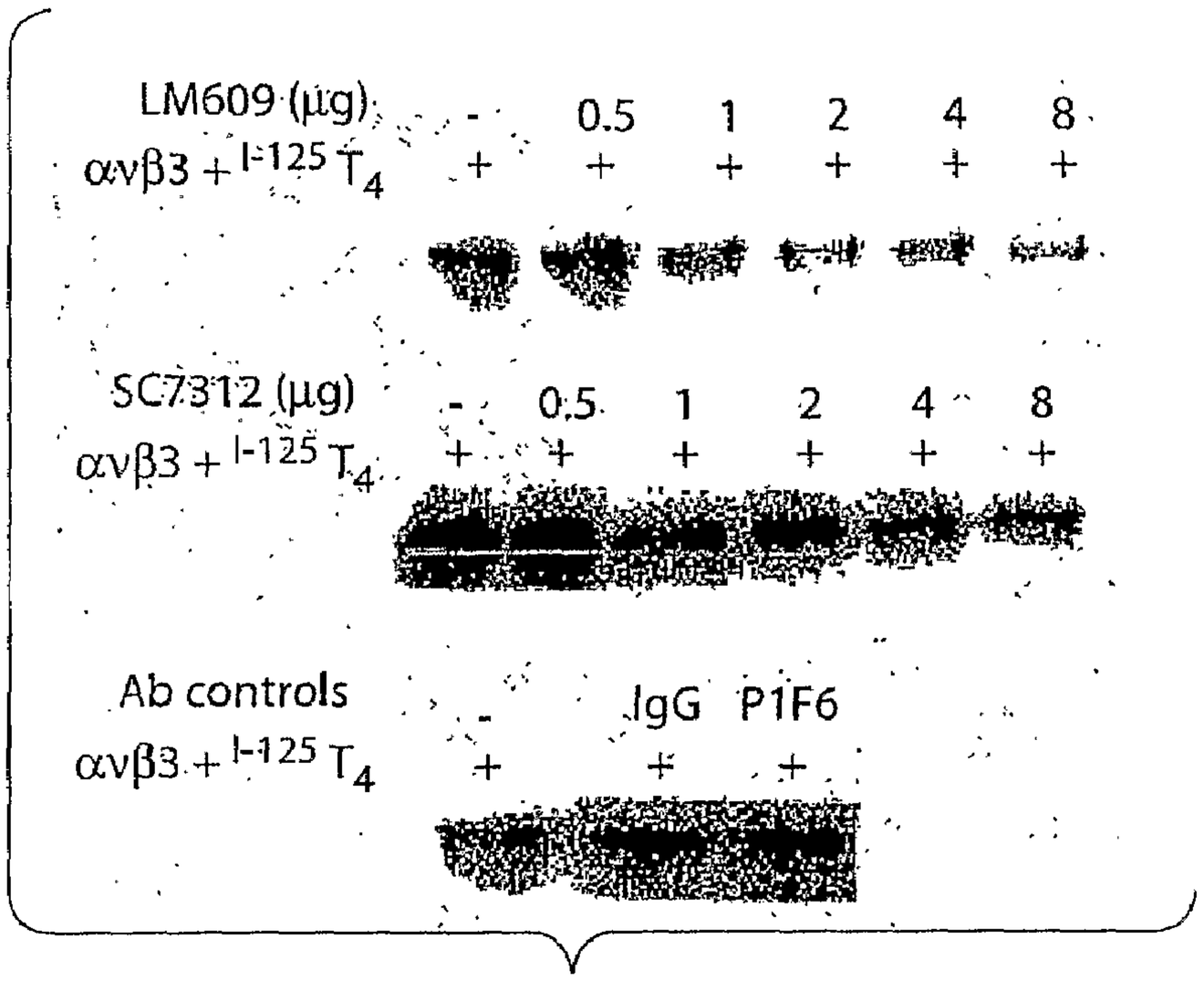


Fig. 26A

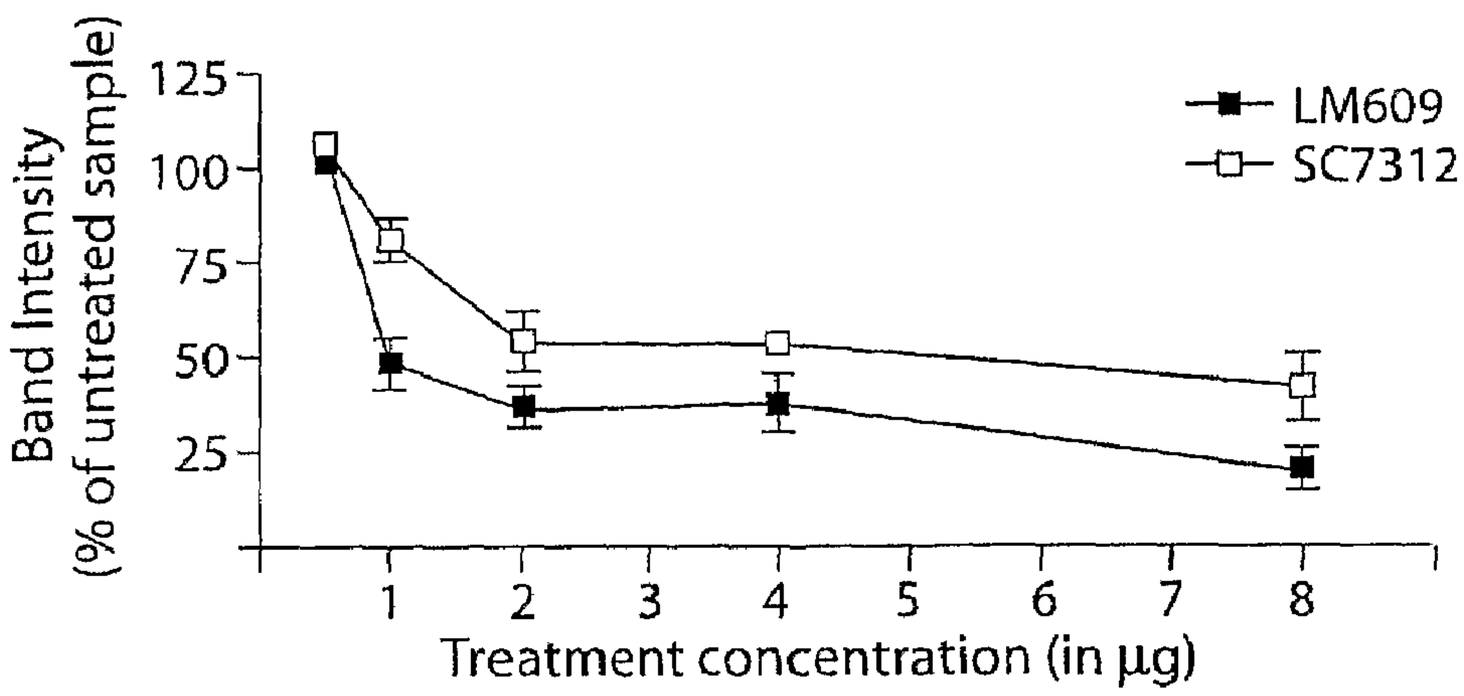


Fig. 26B

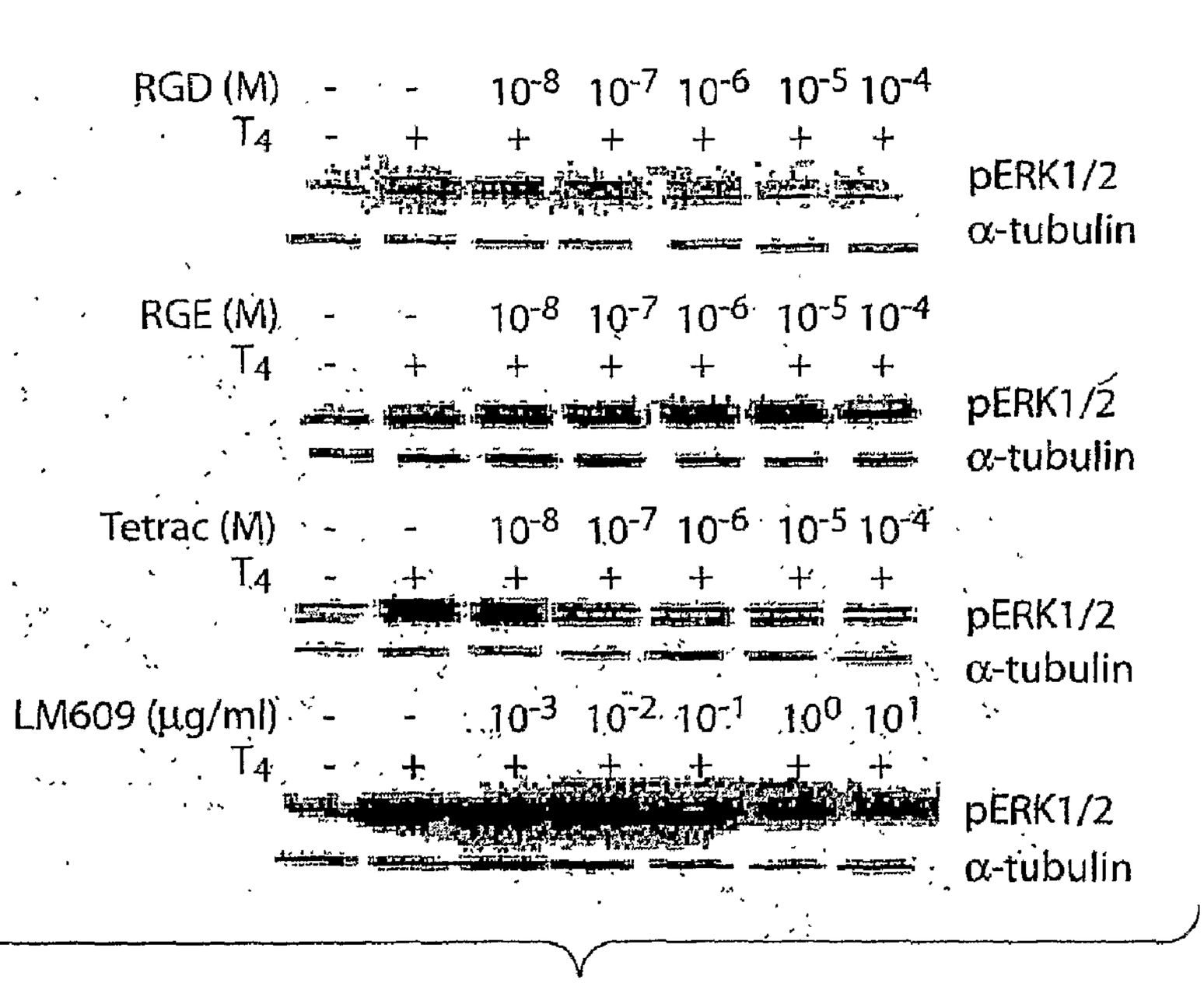


Fig. 27A

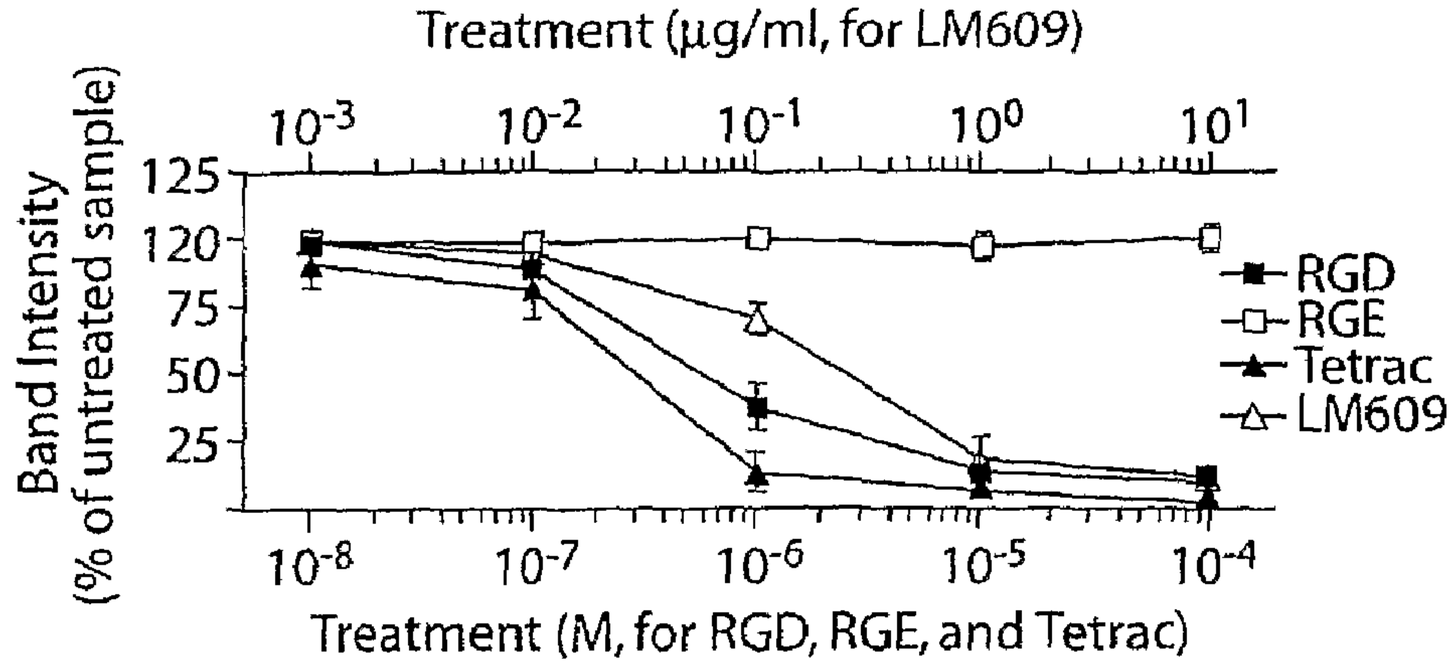


Fig. 27B

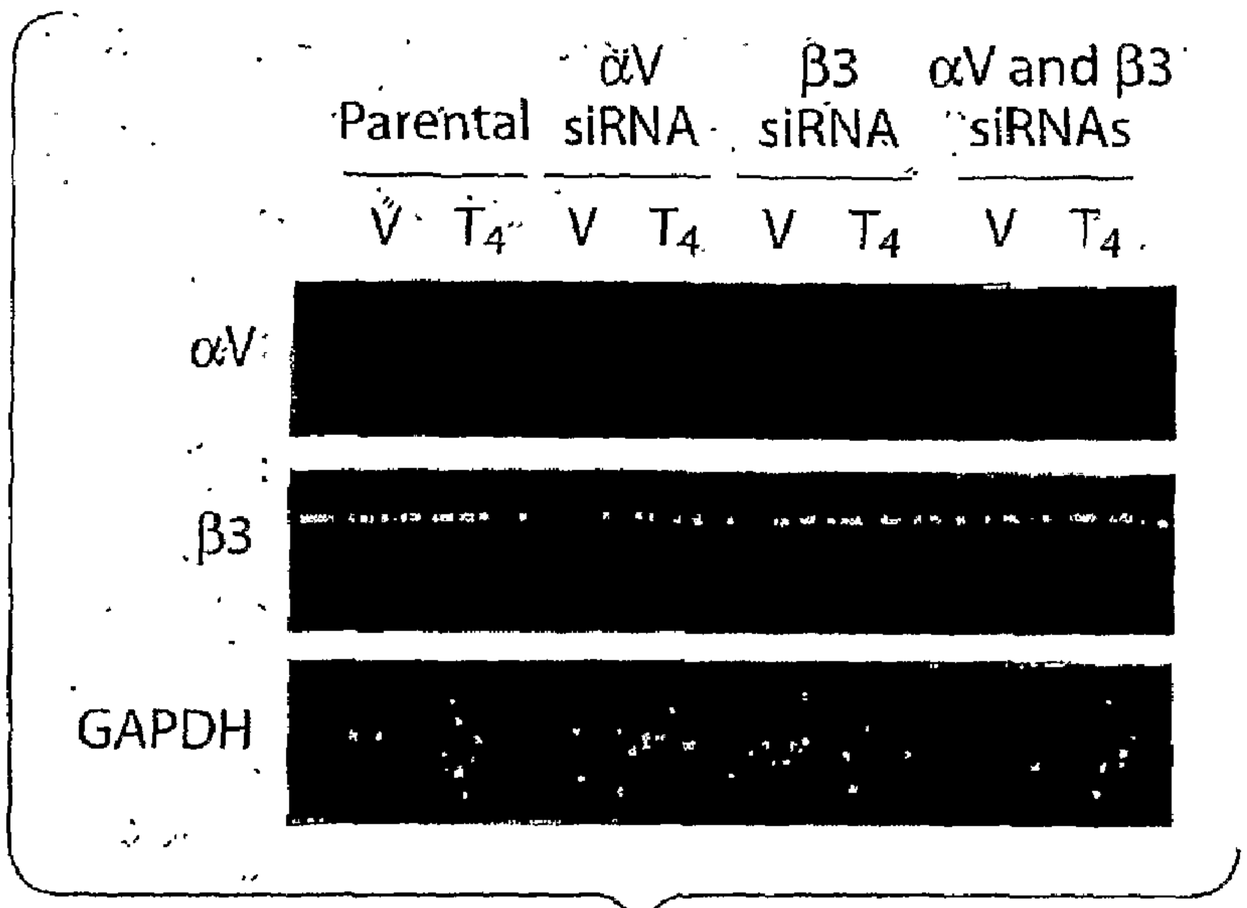


Fig. 28A

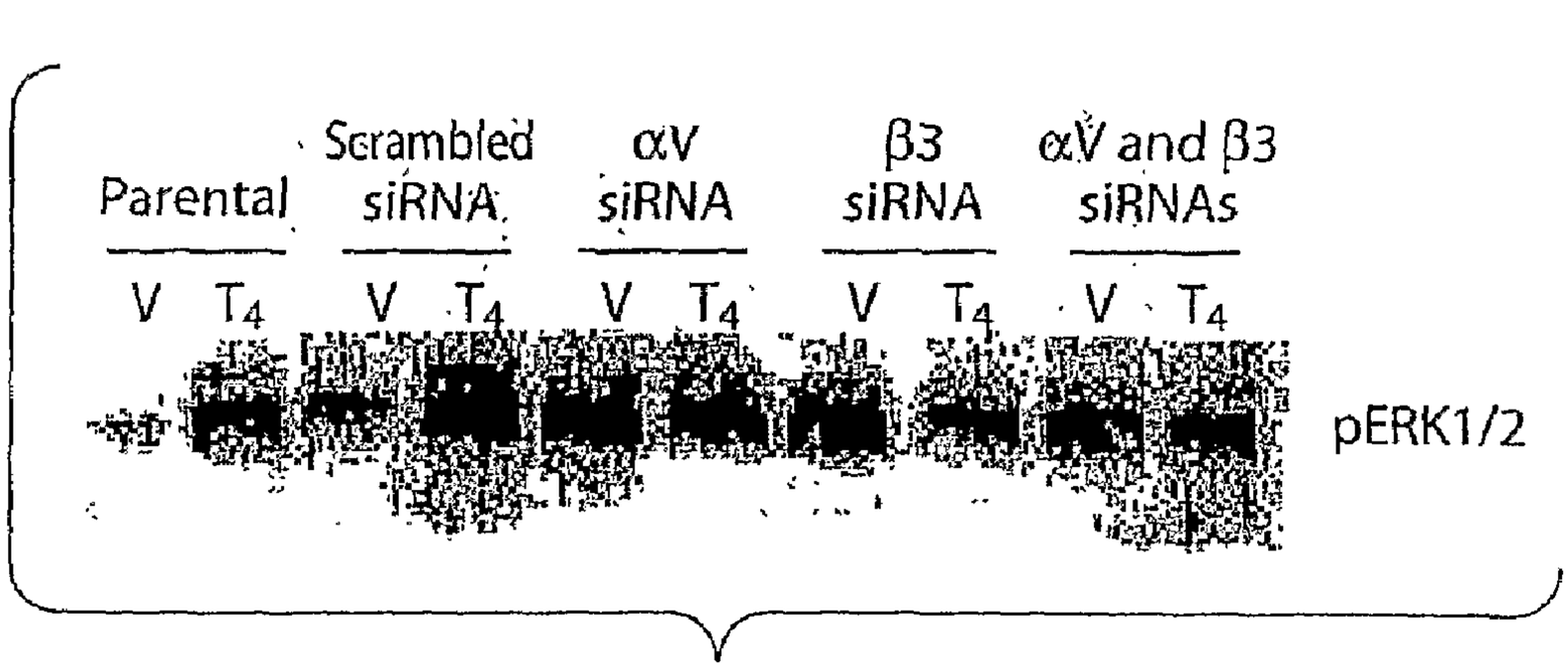


Fig. 28B

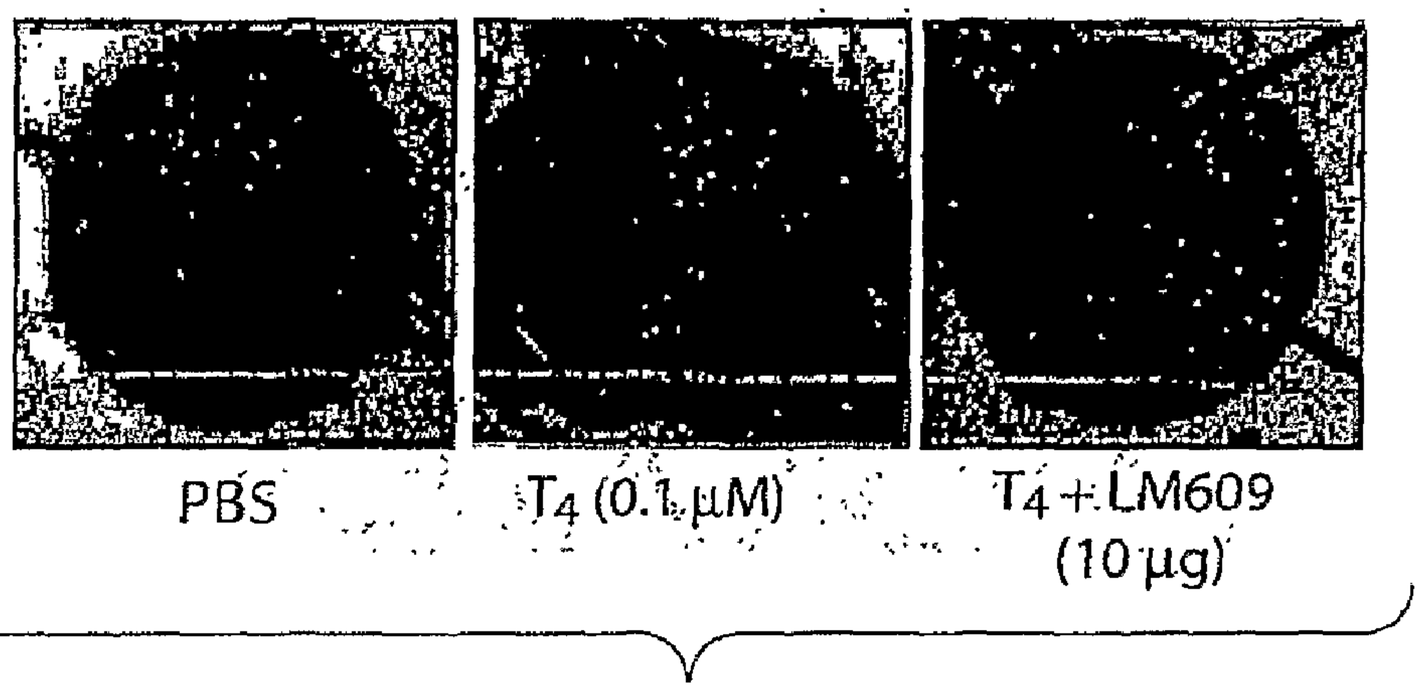


Fig. 29A

CAM Treatment	# of Branches ± SEM	% Inhibition ± SEM
PBS	73 ± 8	-
T_4 (0.1 μM)	170 ± 16	0
T_4 + LM609 (10 μg)	109 ± 9	64 ± 9***

Fig. 29B

THYROID HORMONE ANALOGS AND METHODS OF USE

RELATED APPLICATIONS

This application is the U.S. National Phase application, filed under 35 U.S.C. §371, of PCT/US2005/032813, filed Sep. 15, 2005, which claims priority to U.S. Provisional Application No. 60/670,534, filed Apr. 13, 2005 and is a CIP of U.S. application Ser. No. 10/943,072, filed Sep. 15, 2004, which claims priority to U.S. Provisional Application No. 60/502,721, filed Sep. 15, 2003. Each of these applications is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

This invention relates to thyroid hormone, thyroid hormone analogs and derivatives, and polymeric forms thereof. Methods of using such compounds, and pharmaceutical compositions containing same are also disclosed. The invention also relates to methods of preparing such compounds.

BACKGROUND OF THE INVENTION

Thyroid hormones, L-thyroxine (T4) and L-triiodothyronine (T3), regulate many different physiological processes in different tissues in vertebrates. Most of the actions of thyroid hormones are mediated by the thyroid hormone receptor ("TR"), which is a member of the nuclear receptor superfamily of ligand-activated transcription regulators. This superfamily also includes receptors for steroid hormones, retinoids, and 1,25-dihydroxy vitamin D3. These receptors are transcription factors that can regulate expression of specific genes in various tissues and are targets for widely used drugs, such as tamoxifen, an estrogen receptor partial antagonist. There are two different genes that encode two different TRs, TRα and TRβ. These two TRs are often co-expressed at different levels in different tissues. Most thyroid hormones do not discriminate between the two TRs and bind both with similar affinities.

Gene knockout studies in mice indicate that TRβ plays a role in the development of the auditory system and in the negative feedback of thyroid stimulating hormone by T3 in the pituitary, whereas TRα, modulates the effect of thyroid hormone on calorigenesis and on the cardiovascular system. The identification of TR antagonists could play an important role in the future treatment of hypothyroidism. Such molecules would act rapidly by directly antagonizing the effect of thyroid hormone at the receptor level, a significant improvement for individuals with hypothyroidism who require surgery, have cardiac disease, or are at risk for life-threatening thyrotoxic storm.

Thus, there remains a need for the development of compounds that selectively modulate thyroid hormone action by functioning as isoform-selective agonists or antagonists of the thyroid hormone receptors (TRs) would prove useful for medical therapy. Recent efforts have focused on the design and synthesis of thyroid hormone (T3/T4) antagonists as potential therapeutic agents and chemical probes. There is also a need for the development of thyromimetic compounds that are more accessible than the natural hormone and have potentially useful receptor binding and activation properties.

Thyroid hormone receptor preferentially binds 3,5,3'-triiodo-L-thyronine (T3), a hormone analogue derived by tissue deiodination of circulating L-thyroxine (T4). However, the ability of T4 and T3 to activate intracellular signal transduction cascades, independently of TR, has recently been

described by several laboratories. Acting independently of TR, thyroid hormone also modulates activity of the plasma membrane Na^+/H^+ exchanger, Ca^{2+}-stimulable ATPase, several other ion pumps or channels, and GTPase activity of synaptosomes. Studies from several laboratories have demonstrated the ability of thyroid hormone to activate the MAPK signal transduction cascade. These pathways typically are activated by physical and chemical signals at the cell surface. Although the kinetics and analog specificity for binding of thyroid hormone to the plasma membrane have been repeatedly reported, a cell surface receptor that accounts for these TR-dependent actions for thyroid hormone has not been previously identified.

Our laboratory has shown in the CV-1 monkey fibroblast cell line, which lacks functional TR, and in other cells that T4 activates the mitogen-activated protein kinase (MAPK; ERK112) signaling cascade and promotes the phosphorylation and nuclear translocation of MAPK as early as 10 min following application of a physiological concentration of T4. In nuclear fractions of thyroid hormone-treated cells, we have described complexes of activated MAPK and transactivator nucleoproteins that are substrates for the serine kinase activity of MAPK. These proteins include signal transducer and activator of transcription (STAT)-1α, STAT3, p53, estrogen receptor (ER)-α and, in cells containing TR, the nuclear thyroid hormone receptor for T3 (TRβ1). Thyroid hormone-directed MAPK-dependent phosphorylation of these proteins enhances their transcriptional capabilities. The effects of T4-induced MAPK activation are blocked by inhibitors of the MAPK signal transduction pathway and by tetraiodothyroacetic acid (tetrac), a thyroid hormone analog which inhibits Tq binding to the cell surface. Thyroid hormone-activated MAPK may also act locally at the plasma membrane, e.g., on the N^+/H^+ antiporter, rather than when translocated to the cell nucleus. A cell surface receptor for T4, that is linked to activation of the MAPK cascade has not previously been identified.

Integrins are a family of transmembrane glycoproteins that form noncovalent heterodimers. Extracellular domains of the integrins interact with a variety of ligands, including extracellular matrix glycoproteins, and the intracellular domain is linked to the cytoskeleton. Thyroid hormone was shown a decade ago to influence the interaction of integrin with the extracellular matrix protein, laminin, but the mechanism was not known. Integrin αVβ3 has a large number of extracellular protein ligands, including growth factors, and upon ligand-binding can activate the MAPK cascade. Several of the integrins contain an Arg-Gly-Asp ("RGD") recognition site that is important to the liganding of matrix and other extracellular proteins that contain an Arg-Gly-Asp sequence.

Thus, it would be desirable to identify and provide an initiation site for the induction of MAPK signaling cascades in cells treated with thyroid hormones, or analogs and polymers thereof, thereby providing for methods of modulating growth factors and other polypeptides whose cell surface receptors clustered around this initiation site.

It is estimated that five million people are afflicted with chronic stable angina in the United States. Each year 200,000 people under the age of 65 die with what is termed "premature ischemic heart disease." Despite medical therapy, many go on to suffer myocardial infarction and debilitating symptoms prompting the need for revascularization with either percutaneous transluminal coronary angioplasty or coronary artery bypass surgery. It has been postulated that one way of relieving myocardial ischemia would be to enhance coronary collateral circulation.

Correlations have now been made between the anatomic appearance of coronary collateral vessels ("collaterals") visualized at the time of intracoronary thrombolitic therapy during the acute phase of myocardial infarction and the creatine kinase time-activity curve, infarct size, and aneurysm formation. These studies demonstrate a protective role of collaterals in hearts with coronary obstructive disease, showing smaller infarcts, less aneurysm formation, and improved ventricular function compared with patients in whom collaterals were not visualized. When the cardiac myocyte is rendered ischemic, collaterals develop actively by growth with DNA replication and mitosis of endothelial and smooth muscle cells. Once ischemia develops, these factors are activated and become available for receptor occupation, which may initiate angiogenesis after exposure to exogenous heparin. Unfortunately, the "natural" process by which angiogenesis occurs is inadequate to reverse the ischemia in almost all patients with coronary artery disease.

During ischemia, adenosine is released through the breakdown of ATP. Adenosine participates in many cardio-protective biological events. Adenosine has a role in hemodynamic changes such as bradycardia and vasodilation, and adenosine has been suggested to have a role in such unrelated phenomena as preconditioning and possibly the reduction in reperfusion injury (Ely and Beme, Circulation, 85: 893 (1992).

Angiogenesis is the development of new blood vessels from preexisting blood vessels (Mousa, S. A., In *Angiogenesis Inhibitors and Stimulators: Potential Therapeutic Implications*, Landes Bioscience, Georgetown, Tex.; Chapter 1, (2000)). Physiologically, angiogenesis ensures proper development of mature organisms, prepares the womb for egg implantation, and plays a key role in wound healing. The development of vascular networks during embryogenesis or normal and pathological angiogenesis depends on growth factors and cellular interactions with the extracellular matrix (Breier et al., *Trends in Cell Biology* 6:454-456 (1996); Folkman, *Nature Medicine* 1:27-31 (1995); Risau, *Nature* 386: 671-674 (1997). Blood vessels arise during embryogenesis by two processes: vasculogenesis and angiogenesis (Blood et al., *Bioch. Biophys. Acta* 1032:89-118 (1990). Angiogenesis is a multi-step process controlled by the balance of pro- and anti-angiogenic factors. The latter stages of this process involve proliferation and the organization of endothelial cells (EC) into tube-like structures. Growth factors such as FGF2 and VEGF are thought to be key players in promoting endothelial cell growth and differentiation.

Control of angiogenesis is a complex process involving local release of vascular growth factors (P Carmeliet, Ann NY Acad Sci 902:249-260, 2000), extracellular matrix, adhesion molecules and metabolic factors (R J Tomanek, G C Schatteman, Anat Rec 261:126-135, 2000). Mechanical forces within blood vessels may also play a role (O Hudlicka, Molec Cell Biochem 147:57-68, 1995). The principal classes of endogenous growth factors implicated in new blood vessel growth are the fibroblast growth factor (FGF) family and vascular endothelial growth factor (VEGF) (G Pages, Ann NY Acad Sci 902:187-200, 2000). The mitogen-activated protein kinase (MAPK; ERK1/2) signal transduction cascade is involved both in VEGF gene expression and in control of proliferation of vascular endothelial cells.

Intrinsic adenosine may facilitate the coronary flow response to increased myocardial oxygen demands and so modulate the coronary flow reserve (Ethier et al., Am. J. Physiol., H131 (1993) demonstrated that the addition of physiological concentrations of adenosine to human umbilical vein endothelial cell cultures stimulates proliferation, possibly via a surface receptor. Adenosine may be a factor for human endothelial cell growth and possibly angiogenesis. Angiogenesis appears to be protective for patients with obstructive blood flow such as coronary artery disease ("CAD"), but the rate at which blood vessels grow naturally is inadequate to reverse the disease. Thus, strategies to enhance and accelerate the body's natural angiogenesis potential should be beneficial in patients with CAD.

Similarly, wound healing is a major problem in many developing countries and diabetics have impaired wound healing and chronic inflammatory disorders, with increased use of various cyclooxygenase-2 (CoX2) inhibitors. Angiogenesis is necessary for wound repair since the new vessels provide nutrients to support the active cells, promote granulation tissue formation and facilitate the clearance of debris. Approximately 60% of the granulation tissue mass is composed of blood vessels which also supply the necessary oxygen to stimulate repair and vessel growth. It is well documented that angiogenic factors are present in wound fluid and promote repair while antiangiogenic factors inhibit repair. Wound angiogenesis is a complex multi-step process. Despite a detailed knowledge about many angiogenic factors, little progress has been made in defining the source of these factors, the regulatory events involved in wound angiogenesis and in the clinical use of angiogenic stimulants to promote repair. Further complicating the understanding of wound angiogenesis and repair is the fact that the mechanisms and mediators involved in repair likely vary depending on the depth of the wound, type of wound (burn, trauma, etc.), and the location (muscle, skin, bone, etc.). The condition and age of the patient (diabetic, paraplegic, on steroid therapy, elderly vs infant, etc) can also determine the rate of repair and response to angiogenic factors. The sex of the patient and hormonal status (premenopausal, post menopausal, etc.) may also influence the repair mechanisms and responses. Impaired wound healing particularly affects the elderly and many of the 14 million diabetics in the United States. Because reduced angiogenesis is often a causative agent for wound healing problems in these patient populations, it is important to define the angiogenic factors important in wound repair and to develop clinical uses to prevent and/or correct impaired wound healing.

Thus, there remains a need for an effective therapy in the way of angiogenic agents as either primary or adjunctive therapy for promotion of wound healing, coronary angiogenesis, or other angiogenic-related disorders, with minimum side effects. Such a therapy would be particularly useful for patients who have vascular disorders such as myocardial infarctions, stroke or peripheral artery diseases and could be used prophylactically in patients who have poor coronary circulation, which places them at high risk of ischemia and myocardial infarctions.

Thyroid hormones, analogs, and polymeric conjugations play important roles in the development of the brain. Increasing evidence suggests that the deprivation of polymeric thyroid hormones in the early developmental stage causes structural and functional deficits in the CNS, but the precise mechanism underlying this remains elusive.

The mammalian nervous system comprises a peripheral nervous system (PNS) and a central nervous system (CNS, comprising the brain and spinal cord), and is composed of two principal classes of cells: neurons and glial cells. The glial cells fill the spaces between neurons, nourishing them and modulating their function. Certain glial cells, such as Schwann cells in the PNS and oligodendrocytes in the CNS, also provide a myelin sheath that surrounds neural processes. The myelin sheath enables rapid conduction along the neuron. In the peripheral nervous system, axons of multiple neu-

rons may bundle together in order to form a nerve fiber. These, in turn, may be combined into fascicles or bundles.

During development, differentiating neurons from the central and peripheral nervous systems send out axons that grow and make contact with specific target cells. In some cases, axons must cover enormous distances; some grow into the periphery, whereas others are confined within the central nervous system. In mammals, this stage of neurogenesis is complete during the embryonic phase of life and neuronal cells do not multiply once they have fully differentiated.

A host of neuropathies have been identified that affect the nervous system. The neuropathies, which may affect neurons themselves or associated glial cells, may result from cellular metabolic dysfunction, infection, exposure to toxic agents, autoimmunity, malnutrition, or ischemia. In some cases, the cellular neuropathy is thought to induce cell death directly. In other cases, the neuropathy may induce sufficient tissue necrosis to stimulate the body's immune/inflammatory system and the immune response to the initial injury then destroys neural pathways.

Where the damaged neural pathway results from CNS axonal damage, autologous peripheral nerve grafts have been used to bridge lesions in the central nervous system and to allow axons to make it back to their normal target area. In contrast to CNS neurons, neurons of the peripheral nervous system can extend new peripheral processes in response to axonal damage. This regenerative property of peripheral nervous system axons is thought to be sufficient to allow grafting of these segments to CNS axons. Successful grafting appears to be limited, however, by a number of factors, including the length of the CNS axonal lesion to be bypassed, and the distance of the graft sites from the CNS neuronal cell bodies, with successful grafts occurring near the cell body.

Within the peripheral nervous system, this cellular regenerative property of neurons has limited ability to repair function to a damaged neural pathway. Specifically, the new axons extend randomly, and are often misdirected, making contact with inappropriate targets that can cause abnormal function. For example, if a motor nerve is damaged, regrowing axons may contact the wrong muscles, resulting in paralysis. In addition, where severed nerve processes result in a gap of longer than a few millimeters, e.g., greater than 10 millimeters (mm), appropriate nerve regeneration does not occur, either because the processes fail to grow the necessary distance, or because of misdirected axonal growth. Efforts to repair peripheral nerve damage by surgical means has met with mixed results, particularly where damage extends over a significant distance. In some cases, the suturing steps used to obtain proper alignment of severed nerve ends stimulates the formulation of scar tissue which is thought to inhibit axon regeneration. Even where scar tissue formation has been reduced, as with the use of nerve guidance channels or other tubular prostheses, successful regeneration generally still is limited to nerve damage of less than 10 millimeters in distance. In addition, the reparative ability of peripheral neurons is significantly inhibited where an injury or neuropathy affects the cell body itself or results in extensive degeneration of a distal axon.

Mammalian neural pathways also are at risk due to damage caused by neoplastic lesions. Neoplasias of both the neurons and glial cells have been identified. Transformed cells of neural origin generally lose their ability to behave as normal differentiated cells and can destroy neural pathways by loss of function. In addition, the proliferating tumors may induce lesions by distorting normal nerve tissue structure, inhibiting pathways by compressing nerves, inhibiting cerbrospinal fluid or blood supply flow, and/or by stimulating the body's immune response. Metastatic tumors, which are a significant cause of neoplastic lesions in the brain and spinal cord, also similarly may damage neural pathways and induce neuronal cell death.

One type of morphoregulatory molecule associated with neuronal cell growth, differentiation and development is the cell adhesion molecule ("CAM"), most notably the nerve cell adhesion molecule (N-CAM). The CAMs are members the immunoglobulin super-family. They mediate cell-cell interactions in developing and adult tissues through homophilic binding, i.e., CAM-CAM binding on apposing cells. A number of different CAMs have been identified. Of these, the most thoroughly studied are N-CAM and L-CAM (liver cell adhesion molecules), both of which have been identified on all cells at early stages of development, as well as in different adult tissues. In neural tissue development, N-CAM expression is believed to be important in tissue organization, neuronal migration, nerve-muscle tissue adhesion, retinal formation, synaptogenesis, and neural degeneration. Reduced N-CAM expression also is thought to be associated with nerve dysfunction. For example, expression of at least one form of N-CAM, N-CAM-180, is reduced in a mouse demyelinating mutant. Bhat, Brain Res. 452: 373-377 (1988). Reduced levels of N-CAM also have been associated with normal pressure hydrocephalus, Werdelin, Acta Neurol. Scand. 79: 177-181 (1989), and with type II schizophrenia. Lyons, et al., Biol. Psychiatry 23: 769-775 (1988). In addition, antibodies against N-CAM have been shown to disrupt functional recovery in injured nerves. Remsen, Exp. Neurobiol. 110: 268-273 (1990).

Currently no satisfactory method exists to repair the damage caused by traumatic injuries of motor neurons and diseases of motor neurons. There are 15,000 to 18,000 new cases of spinal cord injury each year in the United States. In addition, there are approximately 200,000 survivors of spinal cord injury. The annual cost of care for these patients exceeds $7 billion. The pathophysiology following acute spinal cord trauma is a complex and not fully understood mechanism. The primary tissue damage caused by mechanical trauma occurs immediately and is irreversible. Allen, J. Am. Med. Assoc. 57: 878-880 (1911). Experimental evidence indicates that much of the post-traumatic tissue damage is the result of a reactive process that begins within minutes after the injury and continues for days or weeks. Janssen, et al., Spine 14: 23-32 (1989) and Panter, et al., (1992). This progressive, self-destructive process includes pathophysiological mechanisms such as hemorrhage, post-traumatic ischemia, edema, axonal and neuronal necrosis, and demyelinization followed by cyst formation and infarction. For review, see Tator, et al., J. Neurosurg, 75: 15-26 (1991) and Faden, Crit. Rev. Neurobiol. 7: 175-186 (1993). Proposed injurious factors include electrolyte changes whereby increased intracellular calcium initiates a cascade of events (Young, J. Neurotrauma 9, Suppl. 1: S9-S25 (1992) and Young, J. Emerg. Med. 11: 13-22 (1993)), biochemical changes with uncontrolled transmitter release (Liu, et al., Cell 66: 807-815 (1991) and Yanase, et al., J. Neurosurg 83: 884-888 (1995), arachidonic acid release, free-radical production, lipid peroxidation (Braughler, et al., J. Neurotrauma 9, Suppl. 1: S1-S7 (1992), eicosanoid production (Demediuk, et al., J. Neurosci. Res. 20: 115-121 (1988), endogenous opioids (Faden, et al., Ann Neurol. 17: 386-390 (1985), metabolic changes including alterations in oxygen and glucose (Faden, Crit. Rev. Neurobiol. 7: 175-186 (1993)), inflammatory changes (Blight, J. Neurotrauma 9, Suppl. 1: S83-S91 (1992), and astrocytic edema (Kimelberg, J. Neurotrauma 9, Suppl. 1: S71-S81 (1992). For the past 400 years surgical approaches including laminectomy and

decompression, accompanied by fusion, have been the most commonly practiced treatment strategies. Hansebout, "Early Management of Acute Spinal Cord Injury", pp. 181-196 (1982) and Janssen, et al., Spine 14: 23-32 (1989). However, these procedures have not involved the application of techniques to augment the regenerative properties of spinal cord tissue.

A host of diseases of motor neurons have been identified, including demyelinating diseases, myelopathies, and diseases of motor neurons such as amyotrophic lateral sclerosis ("ALS"). INTERNAL MEDICINE, ch. 121-123 (4th ed., J. H. Stein, ed., Mosby, 1994). Multiple sclerosis ("MS") is the most common demyelinating disorder of the central nervous system, causing patches of sclerosis (i.e., plaques) in the brain and spinal cord. MS has protean clinical manifestations, depending upon the location and size of the plaque. Typical symptoms include visual loss, diplopia, nystagmus, dysarthria, weakness, paresthesias, bladder abnormalities, and mood alterations. Myriad treatments have been proposed for this long-term variable illness. The list of proposed treatments encompasses everything from diet to electrical stimulation to acupuncture, emotional support, and various forms of immunosupressive therapy. None have proved to be satisfactory.

Progressive loss of lower and upper motor neurons occurs in several diseases (e.g., primary lateral sclerosis, spinal muscular atrophy, benign focal amyotrophy). However, ALS is the most common form of motor neuron disease. Loss of both lower and upper motor neurons occur in ALS. Symptoms include progressive skeletal muscle wasting, weakness, gasciculations, and cramping. Some cases have predominant involvement of brainstem motoneurons (progressive bulbar palsy). Unfortunately, treatment of motor neuron and related disease is largely supportive at this time. INTERNAL MEDICINE, ch. 123 (4th ed., J. H. Stein, ed., Mosby, 1994).

Accordingly, there is a need in the art for treatments of motor neurons disorders and injuries, and related deficits in neural functions. It is, therefore, an object of the present invention to provide compositions and methods for stimulating angiogenesis, for inducing neuronal differentiation, and for preventing the death or degeneration of neuronal cells.

The tyrosines are iodinated at one (monoiodotyrosine) or two (diiodotyrosine) sites and then coupled to form the active hormones (diiodotyrosine+diiodotyrosine→tetraiodothyronine [thyroxine, T_4]; diiodotyrosine+monoiodotyrosine→triiodothyronine [T_3]. Another source of T_3 within the thyroid gland is the result of the outer ring deiodination of T_4 by a selenoenzyme: type I 5'-deiodinase (5'D-I). Thyroglobulin, a glycoprotein containing T_3 and T_4 within its matrix, is taken up from the follicle as colloid droplets by the thyroid cells.

Lysosomes containing proteases cleave T_3 and T_4 from thyroglobulin, resulting in release of free T_3 and T_4. The iodotyrosines (monoiodotyrosine and diiodotyrosine) are also released from thyroglobulin, but only very small amounts reach the bloodstream. Iodine is removed from them by intracellular deiodinases, and this iodine is used by the thyroid gland.

The T_4 and T_3 released from the thyroid by proteolysis reach the bloodstream, where they are bound to thyroid hormone-binding serum proteins for transport. The major thyroid hormone-binding protein is thyroxine-binding globulin ("TBG"), which has high affinity but low capacity for T_4 and T_3. TBG normally accounts for about 75% of the bound hormones. Other thyroid hormone-binding proteins—primarily thyroxine-binding prealbumin, also called transthyretin ("TTR"), which has high affinity but low capacity for T_4, and albumin, which has low affinity but high capacity for T_4 and T_3-account for the remainder of the bound serum thyroid hormones. About 0.03% of the total serum T_4 and 0.3% of the total serum T_3 are free and in equilibrium with the bound hormones. Only free T_4 and T_3 are available to the peripheral tissues for thyroid hormone action.

Thyroid hormones have two major physiologic effects: (1) They increase protein synthesis in virtually every body tissue. (T_3 and T_4 enter cells, where T_3, which is derived from the circulation and from conversion of T_4 to T_3 within the cell, binds to discrete nuclear receptors and influences the formation of mRNA.) (2) T_3 increases O_2 consumption by increasing the activity of the Na^+, K^+-ATPase (Na pump), primarily in tissues responsible for basal O_2 consumption (ie, liver, kidney, heart, and skeletal muscle). The increased activity of Na^+, K^+-ATPase is secondary to increased synthesis of this enzyme; therefore, the increased O_2 consumption is also probably related to the nuclear binding of thyroid hormones. However, a direct effect of T_3 on the mitochondrion has not been ruled out. T_3 is believed to be the active thyroid hormone, although T_4 itself may be biologically active.

The pool of thyroid hormones critical for the biological actions of the hormones is the pool of free thyroid hormone. The size of this pool is determined for short time periods by uptake/release of thyroid hormones into/from cell and binding/release of thyroid hormones by thyroid hormone-binding proteins. Both proportions and absolute concentrations of these proteins differ in blood plasma and cerebrospinal fluid ("CSF"). The most pronounced difference is found for transthyretin ("TTR"), which is the only thyroid hormone-binding plasma protein synthesized in the brain (Schreiber G, Southwell B R, Richardson S J. Hormone delivery systems to the brain-transthyretin. Exp Clin Endocrinol Diabetes. 1995; 103(2):75-80). TTR is also distinct from the other two thyroid hormone-binding plasma proteins in humans by the absence of genetic deficiencies. TTR gene expression was initiated during evolution much earlier in the brain than in the liver. The structure of the domains of TTR involved in thyroxine (TR) T4 binding has been completely conserved for 350 million years. These observations point to a special functional significance of TTR in the brain. It is proposed that this is the determination of the level of free T4 in the extracellular compartment of the brain. T4 can then be converted in the brain to triiodothyronine T3 by specific deiodinases. This T3 can interact with receptors in the cell nuclei, regulating gene transcription.

Alzheimer's disease is a severe neurodegenerative disorder, and currently about 4 million Americans suffer from this disease. As the aging population continues to grow, this number could reach 14 million by the middle of next century unless a cure or prevention is found. At present, there is no sensitive and specific premortem test for early diagnosis of this disease. Alzheimer's disease is currently diagnosed based on the clinical observation of cognitive decline, coupled with the systematic elimination of other possible causes of those symptoms. The confirmation of the clinical diagnosis of "probable Alzheimer's disease" can only be made by examination of the postmortem brain. The Alzheimer's disease brain is characterized by the appearance of two distinct abnormal proteinaceous deposits in regions of the brain responsible for learning and memory (e.g., cerebral cortex and hippocampus). These deposits are extracellular amyloid plaques, which are characteristic of Alzheimer's disease, and intracellular neurofibillary tangles ("NFTs"), which can be found in other neurodegenerative disorders as well. Amyloid peptides are typically either 40 or 42 amino acids in length ("A^{1-40}" or "A^{1-42}", respectively) and are formed from

abnormal processing of a larger membrane-associated protein of unknown function, the amyloid precurser protein ("APP"). Oligomeric aggregates of these peptides are thought to be neurotoxic, eventually resulting in synaptic degeneration and neuronal loss. The amount of amyloid deposition roughly correlates with the severity of symptoms at the time of death.

In the past, there have been several attempts for the design of radiopharmaceuticals that could be used as diagnostic agents for a premortem diagnosis of Alzheimer's disease. Bomebroek et al. showed that the amyloid-associated protein serum amyloid P component (SAP), labeled with ^{123}I, accumulates at low levels in the cerebral cortex, possibly in vessel walls, of patients with cerebral amyloidosis (Bomebroek, M., et al., Nucl. Med. Commun. (1996), Vol. 17, pp. 929-933).

Saito et al. proposed a vector-mediated delivery of ^{123}I-labeled A^{1-40} through the blood-brain barrier. It is reported that the iodinated A^{1-40} binds A amyloid plaque in tissue sections (Saito, Y., et al., Proc. Natl. Acad. Sci. USA 1995, Vol. 92, pp. 10227-10231). U.S. Pat. No. 5,231,000 discloses antibodies with specificity to A4 amyloid polypeptide found in the brain of Alzheimer's disease patients. However, a method to deliver these antibodies across the blood-brain barrier has not been described. Zhen et al. described modifications of the amyloid-binding dye known as "Congo Red™", and complexes of these modified molecules with technetium and rhenium. The complexes with radioactive ions are purported to be potential imaging agents for Alzheimer's disease (Zhen et al., J. Med. Chem. (1999), Vol. 42, pp. 2805-2815). However, the potential of the complexes to cross the blood-brain barrier is limited.

A group at the University of Pennsylvania in the U.S.A. (Skovronsky, M., et al., Proc. Natl. Acad. Sci. 2000, Vol. 97, pp. 7609-7614) has developed a fluorescently labeled derivative of Congo Red that is brain permeable and that non-specifically binds to amyloid materials (that is, peptides in-pleated sheet conformation). This compound would need to be radiolabeled and then run through pre-clinical screens for pharmacokinetics and toxicity before clinical testing. In contrast, our invention utilizes derivatives of naturally occurring substances alone or in combinations for the diagnosis, prevention, and treatment of Alzheimer's disease. Klunk et al. reported experiments with a derivative of Congo Red™, Chrysamine G ("CG"). It is reported that CG binds synthetic-amyloid well in vitro, and crosses the blood-brain barrier in normal mice (Klunk et al., Neurobiol. Aging (1994), Vol. 15, No. 6, pp. 691-698). Bergstrom et al. presented a compound labeled with ^{123}I as a potential radioligand for visualization of M1 and M2 muscarinic acetylcholine receptors in Alzheimer's disease (Bergstrom et al., Eur. J. Nucl. Med. (1999), Vol. 26, pp. 1482-1485).

Recently, it has been discovered that certain specific chemokine receptors are upregulated in the brains of patients with Alzheimer's disease (Horuk, R. et al., J. Immunol. (1997), Vol. 158, pp. 2882-2890); Xia et al., J. Neuro Virol (1999), Vol. 5, pp. 32-41). In addition, it has been recently shown that the chemokine receptor CCR1 is upregulated in the brains of patients with advanced Alzheimer's disease and absent in normal-aged brains (Halks-Miller et al, CCR1 Immunoreactivity in Alzheimer's Disease Brains, Society for Neuroscience Meeting Abstract, #787.6, Volume 24, 1998). Antagonists to the CCR1 receptor and their use as anti-inflammatory agents are described in the PCT Published Patent Application, WO 98/56771.

None of the above described proposals have resulted in a clinical development of an imaging agent for the early diagnosis of Alzheimer's disease. Accordingly, there is still a clinical need for a diagnostic agent that could be used for a reliable and early diagnosis of Alzheimer's disease. Additionally, the proposed strategies would also be useful for the inhibition of amyloid plaque formation or buildup in Alzheimer patients. Accordingly, it is an object of the present invention to provide compositions and methods for the early diagnosis, prevention, and treatment of neurodegenerative diseases, such as, for example Alzheimer's disease.

It is interesting to note that angiogenesis also occurs in other situations, but which are undesirable, including solid tumour growth and metastasis; rheumatoid arthritis; psoriasis; scleroderma; and three common causes of blindness—diabetic retinopathy, retrolental fibroplasia and neovascular glaucoma (in fact, diseases of the eye are almost always accompanied by vascularization. The process of wound angiogenesis actually has many features in common with tumour angiogenesis. Thus, there are some conditions, such as diabetic retinopathy or the occurrence of primary or metastatic tumors, where angiogenesis is undesirable. Thus, there remains a need for methods by which to inhibit the effect of angiogenic agents for the treatment of cancers.

SUMMARY OF THE INVENTION

The invention is based, in part, on the discovery that thyroid hormone, thyroid hormone analogs, and their polymeric forms, act at the cell membrane level and have pro-angiogenic properties that are independent of the nuclear thyroid hormone effects. Accordingly, these thyroid hormone analogs and polymeric forms (i.e., angiogenic agents) can be used to treat a variety of disorders. Similarly, the invention is also based on the discovery that thyroid hormone analog antagonists inhibit the pro-angiogenic effect of such analogs, and can also be used to treat a variety of disorders.

Accordingly, in one aspect the invention features methods for treating a condition amenable to treatment by promoting angiogenesis by administering to a subject in need thereof an amount of a polymeric form of thyroid hormone, or an analog thereof, effective for promoting angiogenesis. Examples of such conditions amenable to treatment by promoting angiogenesis are provided herein and can include occlusive vascular disease, coronary disease, erectile dysfunction, myocardial infarction, ischemia, stroke, peripheral artery vascular disorders, and wounds.

Examples of thyroid hormone analogs are also provided herein and can include triiodothyronine (T3), levothyroxine (T4), 3,5-dimethyl-4-(4'-hydroxy-3'-isopropylbenzyl)-phenoxy acetic acid (GC-1), or 3,5-diiodothyropropionic acid (DITPA), tetraiodothyroacetic acid (TETRAC), and triiodothyroacetic acid (TRIAC). Additional analogs are in FIG. **20** Tables A-D. These analogs can be conjugated to polyvinyl alcohol, acrylic acid ethylene co-polymer, polylactic acid, or agarose. The conjugation is via covalent or non-covalent bonds depending on the polymer used.

In one embodiment the thyroid hormone, thyroid hormone analogs, or polymeric forms thereof are administered by parenteral, oral, rectal, or topical means, or combinations thereof. Parenteral modes of administration include, for example, subcutaneous, intraperitoneal, intramuscular, or intravenous modes, such as by catheter. Topical modes of administration can include, for example, a band-aid.

In another embodiment, the thyroid hormone, thyroid hormone analogs, or polymeric forms thereof can be encapsulated or incorporated in a microparticle, liposome, or polymer. The polymer can include, for example, polyglycolide, polylactide, or co-polymers thereof. The liposome or microparticle has a size of about less than 200 nanometers, and can

be administered via one or more parenteral routes, or another mode of administration. In another embodiment the liposome or microparticle can be lodged in capillary beds surrounding ischemic tissue, or applied to the inside of a blood vessel via a catheter.

Thyroid hormone, thyroid hormone analogs, or polymeric forms thereof according to the invention can also be co-administered with one or more biologically active substances that can include, for example, growth factors, vasodilators, anti-coagulants, anti-virals, anti-bacterials, anti-inflammatories, immuno-suppressants, analgesics, vascularizing agents, or cell adhesion molecules, or combinations thereof. In one embodiment, the thyroid hormone analog or polymeric form is administered as a bolus injection prior to or post-administering one or more biologically active substance.

Growth factors can include, for example, transforming growth factor alpha ("TGFα"), transforming growth factor beta ("TGFβ"), basic fibroblast growth factor, vascular endothelial growth factor, epithelial growth factor, nerve growth factor, platelet-derived growth factor, and vascular permeability factor. Vasodilators can include, for example, adenosine, adenosine derivatives, or combinations thereof. Anticoagulants include, but are not limited to, heparin, heparin derivatives, anti-factor Xa, anti-thrombin, aspirin, clopidgrel, or combinations thereof.

In another aspect of the invention, methods are provided for promoting angiogenesis along or around a medical device by coating the device with a thyroid hormone, thyroid hormone analog, or polymeric form thereof according to the invention prior to inserting the device into a patient. The coating step can further include coating the device with one or more biologically active substance, such as, but not limited to, a growth factor, a vasodilator, an anti-coagulant, or combinations thereof. Examples of medical devices that can be coated with thyroid hormone analogs or polymeric forms according to the invention include stents, catheters, cannulas or electrodes.

In a further aspect, the invention provides methods for treating a condition amenable to treatment by inhibiting angiogenesis by administering to a subject in need thereof an amount of an anti-angiogenesis agent effective for inhibiting angiogenesis. Examples of the conditions amenable to treatment by inhibiting angiogenesis include, but are not limited to, primary or metastatic tumors, diabetic retinopathy, and related conditions. Examples of the anti-angiogenesis agents used for inhibiting angiogenesis are also provided by the invention and include, but are not limited to, tetraiodothyroacetic acid (TETRAC), triiodothyroacetic acid (TRIAC), monoclonal antibody LM609, XT 199 or combinations thereof. Such anti-angiogenesis agents can act at the cell surface to inhibit the pro-angiogenesis agents.

In one embodiment, the anti-angiogenesis agent is administered by a parenteral, oral, rectal, or topical mode, or combination thereof. In another embodiment, the anti-angiogenesis agent can be co-administered with one or more anti-angiogenesis therapies or chemotherapeutic agents.

In yet a further aspect, the invention provides compositions (i.e., angiogenic agents) that include thyroid hormone, and analogs conjugated to a polymer. The conjugation can be through a covalent or non-covalent bond, depending on the polymer. A covalent bond can occur through an ester or anhydride linkage, for example. Examples of the thyroid hormone analogs are also provided by the instant invention and include levothyroxine (T4), triiodothyronine (T3), 3,5-dimethyl-4-(4'-hydroxy-3'-isopropylbenzyl)-phenoxy acetic acid (GC-1), or 3,5-diiodothyropropionic acid (DITPA). In one embodiment, the polymer can include, but is not limited to, polyvinyl alcohol, acrylic acid ethylene co-polymer, polylactic acid, or agarose.

In another aspect, the invention provides for pharmaceutical formulations including the angiogenic agents according to the present invention in a pharmaceutically acceptable carrier. In one embodiment, the pharmaceutical formulations can also include one or more pharmaceutically acceptable excipients.

The pharmaceutical formulations according to the present invention can be encapsulated or incorporated in a liposome, microparticle, or polymer. The liposome or microparticle has a size of less than about 200 nanometers. Any of the pharmaceutical formulations according to the present invention can be administered via parenteral, oral, rectal, or topical means, or combinations thereof. In another embodiment, the pharmaceutical formulations can be co-administered to a subject in need thereof with one or more biologically active substances including, but not limited to, growth factors, vasodilators, anti-coagulants, or combinations thereof.

In other aspects, the present invention concerns the use of the polymeric thyroid hormone analogs and pharmaceutical formulations containing said hormone, for the restoration of neuronal functions and enhancing survival of neural cells. For the purpose of the present invention, neuronal function is taken to mean the collective physiological, biochemical and anatomic mechanisms that allow development of the nervous system during the embryonic and postnatal periods and that, in the adult animal, is the basis of regenerative mechanisms for damaged neurons and of the adaptive capability of the central nervous system when some parts of it degenerate and can not regenerate.

Therefore, the following processes occur in order to achieve neuronal function: denervation, reinnervation, synaptogenesis, synaptic repression, synaptic expansion, the sprouting of axons, neural regeneration, development and organisation of neural paths and circuits to replace the damaged ones. Therefore, the suitable patients to be treated with the polymeric thyroid hormone analogs or combinations thereof according to the present invention are patients afflicted with degenerative pathologies of the central nervous system (senile dementia like Alzheimer's disease, Parkinsonism, Huntington's chorea, cerebellar-spinal adrenoleucodystrophy), trauma and cerebral ischemia.

In a preferred embodiment, methods of the invention for treating motor neuron defects, including amyotrophic lateral sclerosis, multiple sclerosis, and spinal cord injury comprise administering a polymeric thyroid hormone analog, or combinations thereof, and in combination with growth factors, nerve growth factors, or other pro-angiogenesis or neurogenesis factors. Spinal cord injuries include injuries resulting from a tumor, mechanical trauma, and chemical trauma. The same or similar methods are contemplated to restore motor function in a mammal having amyotrophic lateral sclerosis, multiple sclerosis, or a spinal cord injury. Administering one of the aforementioned polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors also provides a prophylactic function. Such administration has the effect of preserving motor function in a mammal having, or at risk of having, amyotrophic lateral sclerosis, multiple sclerosis, or a spinal cord injury. Also according to the invention, polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors administration preserves the integrity of the nigrostriatal pathway.

Specifically, methods of the invention for treating (pre- or post-symptomatically) amyotrophic lateral sclerosis, mul-

tiple sclerosis, or a spinal cord injury comprise administering a polymeric thyroid hormone analog alone or in combination with nerve growth factors or other neurogenesis factors. In a particularly-preferred embodiment, the polymeric thyroid hormone analog alone or in combination with nerve growth factors or other neurogenesis factors is a soluble complex, comprising at least one polymeric thyroid hormone analog alone or in combination with nerve growth factors or other neurogenesis factors.

In one aspect, the invention features compositions and therapeutic treatment methods comprising administering to a mammal a therapeutically effective amount of a morphogenic protein ("polymeric thyroid hormone analog alone or in combination with nerve growth factors or other neurogenesis factors"), as defined herein, upon injury to a neural pathway, or in anticipation of such injury, for a time and at a concentration sufficient to maintain the neural pathway, including repairing damaged pathways, or inhibiting additional damage thereto.

In another aspect, the invention features compositions and therapeutic treatment methods for maintaining neural pathways. Such treatment methods include administering to the mammal, upon injury to a neural pathway or in anticipation of such injury, a compound that stimulates a therapeutically effective concentration of an endogenous polymeric thyroid hormone analog. These compounds are referred to herein as polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors-stimulating agents, and are understood to include substances which, when administered to a mammal, act on tissue(s) or organ(s) that normally are responsible for, or capable of, producing a polymeric thyroid hormone analog alone or in combination with nerve growth factors or other neurogenesis factors and/or secreting a polymeric thyroid hormone analog alone or in combination with nerve growth factors or other neurogenesis factors, and which cause endogenous level of the polymeric thyroid hormone analogs alone or in combination with nerve growth factor or other neurogenesis factors to be altered.

In particular, the invention provides methods for protecting neurons from the tissue destructive effects associated with the body's immune and inflammatory response to nerve injury. The invention also provides methods for stimulating neurons to maintain their differentiated phenotype, including inducing the redifferentiation of transformed cells of neuronal origin to a morphology characteristic of untransformed neurons. In one embodiment, the invention provides means for stimulating production of cell adhesion molecules, particularly nerve cell adhesion molecules ("N-CAM"). The invention also provides methods, compositions and devices for stimulating cellular repair of damaged neurons and neural pathways, including regenerating damaged dendrites or axons. In addition, the invention also provides means for evaluating the status of nerve tissue, and for detecting and monitoring neuropathies by monitoring fluctuations in polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors levels.

In one aspect of the invention, the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors described herein are useful in repairing damaged neural pathways of the peripheral nervous system. In particular, polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors are useful for repairing damaged neural pathways, including transected or otherwise damaged nerve fibers. Specifically, the polymeric thyroid hormone analogs alone or in combination with nerve growth factor or other neurogenesis factors described herein are capable of stimulating complete axonal nerve regeneration, including vascularization and reformation of the myelin sheath. Preferably, the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors are provided to the site of injury in a biocompatible, bioresorbable carrier capable of maintaining the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors at the site and, where necessary, means for directing axonal growth from the proximal to the distal ends of a severed neuron. For example, means for directing axonal growth may be required where nerve regeneration is to be induced over an extended distance, such as greater than 10 mm. Many carriers capable of providing these functions are envisioned. For example, useful carriers include substantially insoluble materials or viscous solutions prepared as disclosed herein comprising laminin, hyaluronic acid or collagen, or other suitable synthetic, biocompatible polymeric materials such as polylactic, polyglycolic or polybutyric acids and/or copolymers thereof. A preferred carrier comprises an extracellular matrix composition derived, for example, from mouse sarcoma cells.

In a particularly preferred embodiment, a polymeric thyroid hormone analog alone or in combination with nerve growth factors or other neurogenesis factors is disposed in a nerve guidance channel which spans the distance of the damaged pathway. The channel acts both as a protective covering and a physical means for guiding growth of a neurite. Useful channels comprise a biocompatible membrane, which may be tubular in structure, having a dimension sufficient to span the gap in the nerve to be repaired, and having openings adapted to receive severed nerve ends. The membrane may be made of any biocompatible, nonirritating material, such as silicone or a biocompatible polymer, such as polyethylene or polyethylene vinyl acetate. The casing also may be composed of biocompatible, bioresorbable polymers, including, for example, collagen, hyaluronic acid, polylactic, polybutyric, and polyglycolic acids. In a preferred embodiment, the outer surface of the channel is substantially impermeable.

The polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors may be disposed in the channel in association with a biocompatible carrier material, or it may be adsorbed to or otherwise associated with the inner surface of the casing, such as is described in U.S. Pat. No. 5,011,486, provided that the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors is accessible to the severed nerve ends.

In another aspect of the invention, polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors described herein are useful to protect against damage associated with the body's immune/inflammatory response to an initial injury to nerve tissue. Such a response may follow trauma to nerve tissue, caused, for example, by an autoimmune dysfunction, neoplastic lesion, infection, chemical or mechanical trauma, disease, by interruption of blood flow to the neurons or glial cells, or by other trauma to the nerve or surrounding material. For example, the primary damage resulting from hypoxia or ischemia-reperfusion following occlusion of a neural blood supply, as in an embolic stroke, is believed to be immunologically associated. In addition, at least part of the damage associated with a number of primary brain tumors also appears to be immunologically related. Application of a polymeric thyroid hormone analog alone or in combination with nerve growth factors or other neurogenesis factors, either directly or systemically alleviates and/or inhibits the immu-

nologically related response to a neural injury. Alternatively, administration of an agent capable of stimulating the expression and/or secretion in vivo of polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors expression, preferably at the site of injury, may also be used. Where the injury is to be induced, as during surgery or other aggressive clinical treatment, the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors or agent may be provided prior to induction of the injury to provide a neuroprotective effect to the nerve tissue at risk.

Generally, polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors useful in methods and compositions of the invention are dimeric proteins that induce morphogenesis of one or more eukaryotic (e.g., mammalian) cells, tissues or organs. Tissue morphogenesis includes de novo or regenerative tissue formation, such as occurs in a vertebrate embryo during development. Of particular interest are polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors that induce tissue-specific morphogenesis at least of bone or neural tissue. As defined herein, a polymeric thyroid hormone analog alone or in combination with nerve growth factor or other neurogenesis factors comprises a pair of polypeptides that, when folded, form a dimeric protein that elicits morphogenetic responses in cells and tissues displaying thyroid receptors. That is, the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors generally induce a cascade of events including all of the following in a morphogenically permissive environment: stimulating proliferation of progenitor cells; stimulating the differentiation of progenitor cells; stimulating the proliferation of differentiated cells; and, supporting the growth and maintenance of differentiated cells. "Progenitor" cells are uncommitted cells that are competent to differentiate into one or more specific types of differentiated cells, depending on their genomic repertoire and the tissue specificity of the permissive environment in which morphogenesis is induced. An exemplary progenitor cell is a hematopoeitic stem cell, a mesenchymal stem cell, a basement epithelium cell, a neural crest cell, or the like. Further, polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors can delay or mitigate the onset of senescence- or quiescence-associated loss of phenotype and/or tissue function. Still further, polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors can stimulate phenotypic expression of a differentiated cell type, including expression of metabolic and/or functional, e.g., secretory, properties thereof. In addition, polymeric thyroid hormone analogs alone or in combination with nerve growth factor or other neurogenesis factors can induce redifferentiation of committed cells (e.g., osteoblasts, neuroblasts, or the like) under appropriate conditions. As noted above, polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors that induce proliferation and/or differentiation at least of bone or neural tissue, and/or support the growth, maintenance and/or functional properties of neural tissue, are of particular interest herein.

Of particular interest are polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors which, when provided to a specific tissue of a mammal, induce tissue-specific morphogenesis or maintain the normal state of differentiation and growth of that tissue. In preferred embodiments, the present polymeric thyroid hormone analog alone or in combination with nerve growth factors or other neurogenesis factors induce the formation of vertebrate (e.g., avian or mammalian) body tissues, such as but not limited to nerve, eye, bone, cartilage, bone marrow, ligament, tooth dentin, periodontium, liver, kidney, lung, heart, or gastrointestinal lining. Preferred methods may be carried out in the context of developing embryonic tissue, or at an aseptic, unscarred wound site in post-embryonic tissue.

Other aspects of the invention include compositions and methods of using thyroid hormone analogs and polymers thereof for imaging and diagnosis of neurodegenerative disorders, such as, for example, Alzheimer's disease. For example, in one aspect, the invention features T4 analogs that have a high specificity for target sites when administered to a subject in vivo. Preferred T4 analogs show a target to non-target ratio of at least 4:1, are stable in vivo and substantially localized to target within 1 hour after administration. In another aspect, the invention features pharmaceutical compositions comprised of a linker attached to the T4 analogs for Technetium, indium for gamma imaging using single photon emission ("SPECT") and with contrast agents for MRI imaging. Additionally, halogenated analogs that bind TTR can inhibit the formation of amyloid fibrils and thus can be utilized for the prevention and treatment of Alzheimer's disease. Such compounds can also be used with positron emission tomography ("PET") imaging methods.

In other aspects, the invention also includes compositions and methods for modulating actions of growth factors and other polypeptides whose cell surface receptors are clustered around integrin $\alpha V\beta 3$, or other cell surface receptors containing the amino acid sequence Arg-Gly-Asp ("RGD"). Polypeptides that can be modulated include, for example, insulin, insulin-like growth factors, epidermal growth factors, and interferon-γ.

The details of one or more embodiments of the invention have been set forth in the accompanying description below. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. Other features, objects, and advantages of the invention will be apparent from the description and from the claims. In the specification and the appended claims, the singular forms include plural references unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. All patents and publications cited in this specification are incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Effects of L-T4 and L-T3 on angiogenesis quantitated in the chick CAM assay. A, Control samples were exposed to PBS and additional samples to 1 nM T3 or 0.1 μmol/L T4 for 3 days. Both hormones caused increased blood vessel branching in these representative images from 3 experiments. B, Tabulation of mean ±SEM of new branches formed from existing blood vessels during the experimental period drawn from 3 experiments, each of which included 9 CAM assays. At the concentrations shown, T3 and T4 caused similar effects (1.9-fold and 2.5-fold increases, respectively, in branch formation). **$P<0.001$ by 1-way ANOVA, comparing hormone-treated with PBS-treated CAM samples.

FIG. **2**. Tetrac inhibits stimulation of angiogenesis by T4 and agarose-linked T4 (T4-ag). A, A 2.5-fold increase in blood vessel branch formation is seen in a representative

CAM preparation exposed to 0.1 μmol/L T4 for 3 days. In 3 similar experiments, there was a 2.3-fold increase. This effect of the hormone is inhibited by tetrac (0.1 μmol/L), a T4 analogue shown previously to inhibit plasma membrane actions of T4.13 Tetrac alone does not stimulate angiogenesis (C). B, T4-ag (0.1 μmol/L) stimulates angiogenesis 2.3-fold (2.9-fold in 3 experiments), an effect also blocked by tetrac. C, Summary of the results of 3 experiments that examine the actions of tetrac, T4-ag, and T4 in the CAM assay. Data (means ±SEM) were obtained from 10 images for each experimental condition in each of 3 experiments. **P<0.001 by ANOVA, comparing T4-treated and T4-agarose-treated samples with PBS-treated control samples.

FIG. **3**. Comparison of the proangiogenic effects of FGF2 and T4. A, Tandem effects of T4 (0.05 μmol/L) and FGF2 (0.5 μg/mL) in submaximal concentrations are additive in the CAM assay and equal the level of angiogenesis seen with FGF2 (1 μg/mL in the absence of T4). B, Summary of results from 3 experiments that examined actions of FGF2 and T4 in the CAM assay (means ±SEM) as in A. *P<0.05; **P<0.001, comparing results of treated samples with those of PBS-treated control samples in 3 experiments.

FIG. **4**. Effect of anti-FGF2 on angiogenesis caused by T4 or exogenous FGF2. A, FGF2 caused a 2-fold increase in angiogenesis in the CAM model in 3 experiments, an effect inhibited by antibody (ab) to FGF2 (8 μg). T4 also stimulated angiogenesis 1.5-fold, and this effect was also blocked by FGF2 antibody, indicating that the action of thyroid hormone in the CAM model is mediated by an autocrine/paracrine effect of FGF2 because T4 and T3 cause FGF2 release from cells in the CAM model (Table 1). We have shown previously that a nonspecific IgG antibody has no effect on angiogenesis in the CAM assay. B, Summary of results from 3 CAM experiments that studied the action of FGF2-ab in the presence of FGF2 or T4. *P<0.01; **P<0.001, indicating significant effects in 3 experiments studying the effects of thyroid hormone and FGF2 on angiogenesis and loss of these effects in the presence of antibody to FGF2.

FIG. **5**. Effect of PD 98059, a MAPK (ERK1/2) signal transduction cascade inhibitor, on angiogenesis induced by T4, T3, and FGF2. A, Angiogenesis stimulated by T4 (0.1 μmol/L) and T3 (1 nmol/L) together is fully inhibited by PD 98059 (3 μmol/L). B, Angiogenesis induced by FGF2 (1 μg/mL) is also inhibited by PD 98059, indicating that the action of the growth factor is also dependent on activation of the ERK1/2 pathway. In the context of the experiments involving T4-agarose (T4-ag) and tetrac (FIG. **2**) indicating that T4 initiates its proangiogenic effect at the cell membrane, results shown in A and B are consistent with 2 roles played by MAPK in the proangiogenic action of thyroid hormone: ERK1/2 transduces the early signal of the hormone that leads to FGF2 elaboration and transduces the subsequent action of FGF2 on angiogenesis. C, Summary of results of 3 experiments, represented by A and B, showing the effect of PD98059 on the actions of T4 and FGF2 in the CAM model. *P<0.01; **P<0.001, indicating results of ANOVA on data from 3 experiments.

FIG. **6**. T4 and FGF2 activate MAPK in ECV304 endothelial cells. Cells were prepared in M199 medium with 0.25% hormone-depleted serum and treated with T4 (0.1 μmol/L) for 15 minutes to 6 hours. Cells were harvested and nuclear fractions prepared as described previously. Nucleoproteins, separated by gel electrophoresis, were immunoblotted with antibody to phosphorylated MAPK (pERK1 and pERK2, 44 and 42 kDa, respectively), followed by a second antibody linked to a luminescence-detection system. A β-actin immunoblot of nuclear fractions serves as a control for gel loading in each part of this figure. Each immunoblot is representative of 3 experiments. A, T4 causes increased phosphorylation and nuclear translocation of ERK1/2 in ECV304 cells. The effect is maximal in 30 minutes, although the effect remains for ≧6 hours. B, ECV304 cells were treated with the ERK1/2 activation inhibitor PD 98059 (PD; 30 μmol/L) or the PKC inhibitor CGP41251 (CGP; 100 nmol/L) for 30 minutes, after which 10^{-7} M T4 was added for 15 minutes to cell samples as shown. Nuclei were harvested, and this representative experiment shows increased phosphorylation (activation) of ERK1/2 by T4 (lane 4), which is blocked by both inhibitors (lanes 5 and 6), suggesting that PKC activity is a requisite for MAPK activation by T4 in endothelial cells. C, ECV304 cells were treated with either T4 (10^{-7} mol/L), FGF2 (10 ng/mL), or both agents for 15 minutes. The figure shows pERK1/2 accumulation in nuclei with either hormone or growth factor treatment and enhanced nuclear pERK1/2 accumulation with both agents together.

FIG. **7**. T4 increases accumulation of FGF2 cDNA in ECV304 endothelial cells. Cells were treated for 6 to 48 hours with T4 (10^{-7} mol/L) and FGF2 and GAPDH cDNAs isolated from each cell aliquot. The levels of FGF2 cDNA, shown in the top blot, were corrected for variations in GAPDH cDNA content, shown in the bottom blot, and the corrected levels of FGF2 are illustrated below in the graph (mean ±SE of mean; n=2 experiments). There was increased abundance of FGF2 transcript in RNA extracted from cells treated with T4 at all time points. *P<0.05; **P<0.01, indicating comparison by ANOVA of values at each time point to control value.

FIG. **8**. 7 Day Chick Embryo Tumor Growth Model. Illustration of the Chick Chorioallantoic Membrane (CAM) model of tumor implant.

FIG. **9**. T4 Stimulates 3D Wound Healing. Photographs of human dermal fibroblast cells exposed to T4 and control, according to the 3D Wound Healing Assay described herein.

FIG. **10**. T4 Dose-Dependently Increases Wound Healing, Day 3. As indicated by the graph, T4 increases wound healing (measured by outmigrating cells) in a dose-dependent manner between concentrations of 0.1 μM and 1.0 μM. This same increase is not seen in concentrations of T4 between 1.0 μM and 3.0 μM.

FIG. **11**. Effect of unlabeled T_4 and T_3 on $^{I\text{-}125}$-T_4 binding to purified integrin. Unlabeled T_4 (10^{-4}M to 10^{-11}M) or T_3 (10^{-4}M to 10^{-8}M) were added to purified αVβ3 integrin (2 μg/sample) and allowed to incubate for 30 min. at room temperature. Two microcuries of 1-125 labeled T_4 was added to each sample. The samples were incubated for 20 min. at room temperature, mixed with loading dye, and run on a 5% Native gel for 24 hrs. at 4° C. at 45 mÅ. Following electrophoresis, the gels were wrapped in plastic wrap and exposed to film. $^{I\text{-}125}$-T_4 binding to purified αVβ3 is unaffected by unlabeled T_4 in the range of 10^{-11}M to 10^{-7}M, but is competed out in a dose-dependent manner by unlabeled T_4 at a concentration of 10^{-6}M. Hot T_4 binding to the integrin is almost completely displaced by 10^{4}M unlabeled T_4. T_3 is less effective at competing out T_4 binding to αVβ3, reducing the signal by 11%, 16%, and 28% at 10^{-6}M, 10^{-5}M, and 10^{-4}M T_3, respectively.

FIG. **12**. Tetrac and an RGD containing peptide, but not an RGE containing peptide compete out T_4 binding to purified αVβ3. A) Tetrac addition to purified αVβ3 reduces $^{I\text{-}125}$-labeled T_4 binding to the integrin in a dose dependent manner. 10^{-8}M tetrac is ineffective at competing out hot T_4 binding to the integrin. The association of T_4 and αVβ3 was reduced by 38% in the presence of 10^{-7}M tetrac and by 90% with 10^{-5}M tetrac. Addition of an RGD peptide at 10^{-5}M competes out T_4 binding to αVβ3. Application of 10^{-5}M and 10^{-4}M RGE

peptide, as a control for the RGD peptide, was unable to diminish hot T_4 binding to purified αVβ3. B) Graphical representation of the tetrac and RGD data from panel A. Data points are shown as the mean ±S.D. for 3 independent experiments.

FIG. **13**. Effects of the monoclonal antibody LM609 on T_4 binding to αVβ3. A) LM609 was added to αVβ3 at the indicated concentrations. One μg of LM609 per sample reduces labeled T_4 binding to the integrin by 52%. Maximal inhibition of T_4 binding to the integrin is reached when concentrations of LM609 are 2 μg per sample and is maintained with antibody concentrations as high as 8 μg. As a control for antibody specificity, 10 μg/sample Cox-2 mAB and 10 μg/sample mouse IgG were added to αVβ3 prior to incubation with T_4 B) Graphical representation of data from panel A. Data points are shown as the mean ±S.D. for 3 independent experiments.

FIG. **14**. Effect of RGD, RGE, tetrac, and the mAB LM609 on T_4-induced MAPK activation. A) CV-1 cells (50-70% confluency) were treated for 30 min. with 10^{-7} M T_4 (10^{-7} M total concentration, 10^{-10}M free concentration. Selected samples were treated for 16 hrs with the indicated concentrations of either an RGD containing peptide, an RGE containing peptide, tetrac, or LM609 prior to the addition of T_4. Nuclear proteins ere separated by SDS-PAGE and immunoblotted with anti-phospho-MAPK (pERK1/2) antibody. Nuclear accumulation of pERK1/2 is diminished in samples treated with 10^{-6} M RGD peptide or higher, but not significantly altered in samples treated with 10^{-4} M RGE. pERK1/2 accumulation is decreased 76% in CV1 cells treated with 10^{-6}M tetrac, while 10^{-5}M and higher concentrations of tetrac reduce nuclear accumulation of pERK1/2 to levels similar to the untreated control samples. The monoclonal antibody to αVβ3 LM609 decrease accumulation of activated MAPK in the nucleus when it is applied to CV1 cultures a concentration of 1 μg/ml. B) Graphical representation of the data for RGD, RGE, and tetrac shown in panel A. Data points represent the mean ±S.D. for 3 separate experiments.

FIG. **15**. Effects of siRNA to αV and p3 on T_4 induced MAPK activation. CV1 cells were transfected with siRNA (100 nM final concentration) to αV, β3, or αV and β3 together. Two days after transfection, the cells were treated with 10^7M T_4. A) RT-PCR was performed from RNA isolated from each transfection group to verify the specificity and functionality of each siRNA. B) Nuclear proteins from each transfection were isolated and subjected to SDS-PAGE.

FIG. **16**. Inhibitory Effect of αVβ3 mAB (LM609) on T_4-stimulated Angiogenesis in the CAM Model. A) Samples were exposed to PBS, T_4 (0.1 μM), or T_4 plus 10 mg/ml LM609 for 3 days. Angiogenesis stimulated by T_4 is substantially inhibited by the addition of the αVβ3 monoclonal antibody LM609. B) Tabulation of the mean ±SEM of new branches formed from existing blood vessels during the experimental period. Data was drawn from 3 separate experiments, each containing 9 samples in each treatment group. C, D) Angiogenesis stimulated by T4 or FGF2 is also inhibited by the addition of the αVβ3 monoclonal antibody LM609 or XT 199.

FIG. **17**. Polymer Compositions of Thyroid Hormone Analogs—Polymer Conjugation Through an Ester Linkage Using Polyvinyl Alcohol. In this preparation commercially available polyvinyl alcohol (or related co-polymers) can be esterified by treatment with the acid chloride of thyroid hormone analogs, namely the acid chloride form. The hydrochloride salt is neutralized by the addition of triethylamine to afford triethylamine hydrochloride which can be washed away with water upon precipitation of the thyroid hormone ester polymer form for different analogs. The ester linkage to the polymer may undergo hydrolysis in vivo to release the active pro-angiogenesis thyroid hormone analog.

FIG. **18**. Polymer Compositions of Thyroid Hormone Analogs—Polymer Conjugation Through an Anhydride Linkage Using Acrylic Acid Ethylene Co-polymer. This is similar to the previous polymer covalent conjugation however this time it is through an anhydride linkage that is derived from reaction of an acrylic acid co-polymer. This anhydride linkage is also susceptible to hydrolysis in vivo to release thyroid hormone analog. Neutralization of the hydrochloric acid is accomplished by treatment with triethylamine and subsequent washing of the precipitated polyanhydride polymer with water removes the triethylamine hydrochloride byproduct. This reaction will lead to the formation of Thyroid hormone analog acrylic acid co-polymer+triethylamine. Upon in vivo hydrolysis, the thyroid hormone analog will be released over time that can be controlled plus acrylic acid ethylene Co-polymer.

FIG. **19**. Polymer Compositions of Thyroid Hormone Analogs—Entrapment in a Polylactic Acid Polymer. Polylactic acid polyester polymers (PLA) undergo hydrolysis in vivo to the lactic acid monomer and this has been exploited as a vehicle for drug delivery systems in humans. Unlike the prior two covalent methods where the thyroid hormone analog is linked by a chemical bond to the polymer, this would be a non-covalent method that would encapsulate the thyroid hormone analog into PLA polymer beads. This reaction will lead to the formation of Thyroid hormone analog containing PLA beads in water. Filter and washing will result in the formation of thyroid hormone analog containing PLA beads, which upon in vivo hydrolysis will lead to the generation of controlled levels of thyroid hormone plus lactic acid.

FIG. **20**. Thyroid Hormone Analogs Capable of Conjugation with Various Polymers. A-D show substitutions required to achieve various thyroid hormone analogs which can be conjugated to create polymeric forms of thyroid hormone analogs of the invention.

FIG. **21**. In vitro 3-D Angiogenesis Assay FIG. **21** is a protocol and illustration of the three-dimensional in vitro sprouting assay for human micro-vascular endothelial on fibrin-coated beads.

FIG. **22**. In Vitro Sprout Angiogenesis of HOMEC in 3-D Fibrin FIG. **22** is an illustration of human micro-vascular endothelial cell sprouting in three dimensions under different magnifications

FIGS. **23**A-E. Release of platelet-derived wound healing factors in the presence of low level collagen

FIGS. **24**A-B. Unlabeled T4 and T3 displace [^{125}I]-T4 from purified integrin. Unlabeled T4 (10^{-11} M to 10^{-4} M) or T3 (10^{-8} to 10^{-4} M) were added to purified αVβ3 integrin (2 μg/sample) prior to the addition of [^{125}I]-T4. (a) [^{125}I]-T4 binding to purified αVβ3 was unaffected by unlabeled T4 in the range of 10^{-11} M to 10^{-7} M, but was displaced in a concentration-dependent manner by unlabeled T4 at concentrations≧10^{-6} M. T3 was less effective at displacing T4 binding to αVβ3. (b) Graphic presentation of the T4 and T3 data shows the mean ±S.D. of 3 independent experiments.

FIGS. **25**A-B. Tetrac and an RGD-containing peptide, but not an RGE-containing peptide, displace T4 binding to purified αVβ3. (a) Pre-incubation of purified αVβ3 with tetrac or an RGD-containing peptide reduced the interaction between the integrin and [^{125}I]-T4 in a dose-dependent manner. Application of 10^{-5} M and 10^{-4} M RGE peptide, as controls for the RGD peptide, did not diminish labeled T4 binding to purified

αVβ3. (b) Graphic presentation of the tetrac and RGD data indicates the mean ±S.D. of results from 3 independent experiments.

FIGS. **26**A-B. Integrin antibodies inhibit T4 binding to αVβ3. The antibodies LM609 and SC7312 were added to αVβ3 at the indicated concentrations (μg/ml) 30 min prior to the addition of [^{125}I]-T4. Maximal inhibition of T4 binding to the integrin was reached when the concentration of LM609 was 2 μg/ml and was maintained with antibody concentrations as high as 8 μg/ml. SC7312 reduced T4 binding to αVβ3 by 46% at 2 μg/ml antibody/sample and by 58% when 8 μg/ml of antibody were present. As a control for antibody specificity, 10 μg/ml of anti-αVβ3 mAb (P1F6) and 10 μg/ml mouse IgG were added to αVβ3 prior to incubation with T4. The graph shows the mean ±S.D. of data from 3 independent experiments.

FIGS. **27**A-B. Effect of RGD and RGE peptides, tetrac, and the mAb LM609 on T4-induced MAPK activation. (a) Nuclear accumulation of pERK1/2 was diminished in samples treated with 10^{-6} M RGD peptide or higher, but not significantly altered in samples treated with up to 10^{-4} M RGE. pERK1/2 accumulation in CV-1 cells treated with 10^{-5} M tetrac and T4 were similar to levels observed in the untreated control samples. LM609, a monoclonal antibody to αVβ3, decreased accumulation of activated MAPK in the nucleus when it was applied to CV-1 cultures in a concentration of 1 μg/ml. (b) The graph shows the mean ±S.D. of data from 3 separate experiments. Immunoblots with α-tubulin antibody are included as gel-loading controls.

FIGS. **28**A-B. Effects of siRNA to αV and β3 on T4-induced MAPK activation. CV-1 cells were transfected with siRNA (100 nM final concentration) to αV, β3, or αV and β3 together. Two days after transfection, the cells were treated with 10-7 M T4 or the vehicle control for 30 min. (a) RT-PCR was performed with RNA isolated from each transfection group to verify the specificity and functionality of each siRNA. (b) Nuclear proteins from each set of transfected cells were isolated, subjected to SDS-PAGE, and probed for pERK1/2 in the presence or absence of treatment with T4. In the parental cells and in those treated with scrambled siRNA, nuclear accumulation of pERK1/2 with T4 was evident. Cells treated with siRNA to αV or β3 showed an increase in pERK1/2 in the absence of T4, and a decrease with T4 treatment. Cells containing αV and β3 siRNAs did not respond to T4 treatment.

FIGS. **29**A-B. Inhibitory effect of αVβ3 mAb (LM609) on T4-stimulated angiogenesis in the CAM model. CAMS were exposed to filter disks treated with PBS, T4 (10-7 M), or T4 plus 10 μg/ml LM609 for 3 days. (a) Angiogenesis stimulated by T4 was substantially inhibited by the addition of the αVβ3 monoclonal antibody LM609. (b) Tabulation of the mean ±SEM of new branches formed from existing blood vessels during the experimental period is shown. ***P<0.001, comparing results of T4/LM609-treated samples with T4-treated samples in 3 separate experiments, each containing 9 images per treatment group. Statistical analysis was performed by 1-way ANOVA.

DETAILED DESCRIPTION OF THE INVENTION

The features and other details of the invention will now be more particularly described with references to the accompanying drawings, and as pointed out by the claims. For convenience, certain terms used in the specification, examples and claims are collected here. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains.

As used herein, the term "angiogenic agent" includes any compound or substance that promotes or encourages angiogenesis, whether alone or in combination with another substance. Examples include, but are not limited to, T3, T4, T3 or T4-agarose, polymeric analogs of T3, T4, 3,5-dimethyl-4-(4'-hydroxy-3'-isopropylbenzyl)-phenoxy acetic acid (GC-1), or DITPA. In contrast, the terms "anti-angiogenesis agent" or anti-angiogenic agent" refer to any compound or substance that inhibits or discourages angiogenesis, whether alone or in combination with another substance. Examples include, but are not limited to, TETRAC, TRIAC, XT 199, and mAb LM609.

As used herein, the term "myocardial ischemia" is defined as an insufficient blood supply to the heart muscle caused by a decreased capacity of the heart vessels. As used herein, the term "coronary disease" is defined as diseases/disorders of cardiac function due to an imbalance between myocardial function and the capacity of coronary vessels to supply sufficient blood flow for normal function. Specific coronary diseases/disorders associated with coronary disease which can be treated with the compositions and methods described herein include myocardial ischemia, angina pectoris, coronary aneurysm, coronary thrombosis, coronary vasospasm, coronary artery disease, coronary heart disease, coronary occlusion and coronary stenosis.

As used herein the term "occlusive peripheral vascular disease" (also known as peripheral arterial occlusive disorder) is a vascular disorder-involving blockage in the carotid or femoral arteries, including the iliac artery. Blockage in the femoral arteries causes pain and restricted movement. A specific disorder associated with occlusive peripheral vascular disease is diabetic foot, which affects diabetic patients, often resulting in amputation of the foot.

As used herein the terms "regeneration of blood vessels," "angiogenesis," "revascularization," and "increased collateral circulation" (or words to that effect) are considered as synonymous. The term "pharmaceutically acceptable" when referring to a natural or synthetic substance means that the substance has an acceptable toxic effect in view of its much greater beneficial effect, while the related the term, "physiologically acceptable," means the substance has relatively low toxicity. The term, "co-administered" means two or more drugs are given to a patient at approximately the same time or in close sequence so that their effects run approximately concurrently or substantially overlap. This term includes sequential as well as simultaneous drug administration.

"Pharmaceutically acceptable salts" refers to pharmaceutically acceptable salts of thyroid hormone analogs, polymeric forms, and derivatives, which salts are derived from a variety of organic and inorganic counter ions well known in the art and include, by way of example only, sodium, potassium, calcium, magnesium, ammonium, tetra-alkyl ammonium, and the like; and when the molecule contains a basic functionality, salts of organic or inorganic acids, such as hydrochloride, hydrobromide, tartrate, mesylate, acetate, maleate, oxalate and the like can be used as the pharmaceutically acceptable salt. The term also includes both acid and base addition salts.

"Pharmaceutically acceptable acid addition salt" refers to those salts which retain the biological effectiveness and properties of the free bases, which are not biologically or otherwise undesirable, and which are formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid,

nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, pyruvic acid, maleic acid, malonic acid, succinic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, mandelic acid, methanesulfonic acid, ethanesulfonic acid, p-toluenesulfonic acid, salicylic acid, and the like. Particularly preferred salts of compounds of the invention are the monochloride salts and the dichloride salts.

"Pharmaceutically acceptable base addition salt" refers to those salts which retain the biological effectiveness and properties of the free acids, which are not biologically or otherwise undesirable. These salts are prepared from addition of an inorganic base or an organic base to the free acid. Salts derived from inorganic bases include, but are not limited to, the sodium, potassium, lithium, ammonium, calcium, magnesium, zinc, aluminum salts and the like. Preferred inorganic salts are the ammonium, sodium, potassium, calcium, and magnesium salts. Salts derived from organic bases include, but are not limited to, salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, ethanolamine, 2-dimethylaminoethanol, 2-diethylaminoethanol, trimethamine, dicyclohexylamine, lysine, arginine, histidine, caffeine, procaine, hydrabamine, choline, betaine, ethylenediamine, glucosamine, methylglucamine, theobromine, purines, piperazine, piperidine, N-ethylpiperidine, polyamine resins and the like. Particularly preferred organic bases are isopropylamine, diethylamine, ethanolamine, trimethylamine, dicyclohexylamine, choline and caffeine.

"Ureido" refers to a radical of the formula —N(H)—C(O)—NH_2.

It is understood from the above definitions and examples that for radicals containing a substituted alkyl group any substitution thereon can occur on any carbon of the alkyl group. The compounds of the invention, or their pharmaceutically acceptable salts, may have asymmetric carbon atoms in their structure. The compounds of the invention and their pharmaceutically acceptable salts may therefore exist as single enantiomers, diastereoisomers, racemates, and mixtures of enantiomers and diastereomers. All such single enantiomers, diastereoisomers, racemates and mixtures thereof are intended to be within the scope of this invention. Absolute configuration of certain carbon atoms within the compounds, if known, are indicated by the appropriate absolute descriptor R or S.

Separate enantiomers can be prepared through the use of optically active starting materials and/or intermediates or through the use of conventional resolution techniques, e.g., enzymatic resolution or chiral HPLC.

As used herein, the phrase "growth factors" or "neurogenesis factors" refers to proteins, peptides or other molecules having a growth, proliferative, differentiative, or trophic effect on cells of the CNS or PNS. Such factors may be used for inducing proliferation or differentiation and can include, for example, any trophic factor that allows cells of the CNS or PNS to proliferate, including any molecule which binds to a receptor on the surface of the cell to exert a trophic, or growth-inducing effect on the cell. Preferred factors include, but are not limited to, nerve growth factor ("NGF"), epidermal growth factor ("EGF"), platelet-derived growth factor ("PDGF"), insulin-like growth factor ("IGF"), acidic fibroblast growth factor ("aFGF" or "FGF-1"), basic fibroblast growth factor ("bFGF" or "FGF-2"), and transforming growth factor-alpha and -beta ("TGF-α" and "TGF-β").

"Subject" includes living organisms such as humans, monkeys, cows, sheep, horses, pigs, cattle, goats, dogs, cats, mice, rats, cultured cells therefrom, and transgenic species thereof. In a preferred embodiment, the subject is a human. Administration of the compositions of the present invention to a subject to be treated can be carried out using known procedures, at dosages and for periods of time effective to treat the condition in the subject. An effective amount of the therapeutic compound necessary to achieve a therapeutic effect may vary according to factors such as the age, sex, and weight of the subject, and the ability of the therapeutic compound to treat the foreign agents in the subject. Dosage regimens can be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

"Administering" includes routes of administration which allow the compositions of the invention to perform their intended function, e.g., promoting angiogenesis. A variety of routes of administration are possible including, but not necessarily limited to parenteral (e.g., intravenous, intra-arterial, intramuscular, subcutaneous injection), oral (e.g., dietary), topical, nasal, rectal, or via slow releasing microcarriers depending on the disease or condition to be treated. Oral, parenteral and intravenous administration are preferred modes of administration. Formulation of the compound to be administered will vary according to the route of administration selected (e.g., solution, emulsion, gels, aerosols, capsule). An appropriate composition comprising the compound to be administered can be prepared in a physiologically acceptable vehicle or carrier and optional adjuvants and preservatives. For solutions or emulsions, suitable carriers include, for example, aqueous or alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media, sterile water, creams, ointments, lotions, oils, pastes and solid carriers. Parenteral vehicles can include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles can include various additives, preservatives, or fluid, nutrient or electrolyte replenishers (See generally, *Remington's Pharmaceutical Science,* 16th Edition, Mack, Ed. (1980)).

"Effective amount" includes those amounts of pro-angiogenic or anti-angiogenic compounds which allow it to perform its intended function, e.g., promoting or inhibiting angiogenesis in angiogenesis-related disorders as described herein. The effective amount will depend upon a number of factors, including biological activity, age, body weight, sex, general health, severity of the condition to be treated, as well as appropriate pharmacokinetic properties. For example, dosages of the active substance may be from about 0.01 mg/kg/day to about 500 mg/kg/day, advantageously from about 0.1 mg/kg/day to about 100 mg/kg/day. A therapeutically effective amount of the active substance can be administered by an appropriate route in a single dose or multiple doses. Further, the dosages of the active substance can be proportionally increased or decreased as indicated by the exigencies of the therapeutic or prophylactic situation.

"Pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like which are compatible with the activity of the compound and are physiologically acceptable to the subject. An example of a pharmaceutically acceptable carrier is buffered normal saline (0.15M NaCl). The use of such media and

agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the therapeutic compound, use thereof in the compositions suitable for pharmaceutical administration is contemplated. Supplementary active compounds can also be incorporated into the compositions.

"Additional ingredients" include, but are not limited to, one or more of the following: excipients; surface active agents; dispersing agents; inert diluents; granulating and disintegrating agents; binding agents; lubricating agents; sweetening agents; flavoring agents; coloring agents; preservatives; physiologically degradable compositions such as gelatin; aqueous vehicles and solvents; oily vehicles and solvents; suspending agents; dispersing or wetting agents; emulsifying agents, demulcents; buffers; salts; thickening agents; fillers; emulsifying agents; antioxidants; antibiotics; antifungal agents; stabilizing agents; and pharmaceutically acceptable polymeric or hydrophobic materials. Other "additional ingredients" which may be included in the pharmaceutical compositions of the invention are known in the art and described, e.g., in *Remington's Pharmaceutical Sciences*.

Compositions

Disclosed herein are angiogenic agents comprising thyroid hormones, analogs thereof, and polymer conjugations of the hormones and their analogs. The disclosed compositions can be used for promoting angiogenesis to treat disorders wherein angiogenesis is beneficial. Additionally, the inhibition of these thyroid hormones, analogs and polymer conjugations can be used to inhibit angiogenesis to treat disorders associated with such undesired angiogenesis. As used herein, the term "angiogenic agent" includes any compound or substance that promotes or encourages angiogenesis, whether alone or in combination with another substance. Examples include, but are not limited to, T3, T4, T3 or T4-agarose, polymeric analogs of T3, T4, 3,5-dimethyl-4-(4'-hydroxy-3'-isopropylbenzyl)-phenoxy acetic acid (GC-1), or DITPA.

Polymer conjugations are used to improve drug viability. While many old and new therapeutics are well-tolerated, many compounds need advanced drug discovery technologies to decrease toxicity, increase circulatory time, or modify biodistribution. One strategy for improving drug viability is the utilization of water-soluble polymers. Various water-soluble polymers have been shown to modify biodistribution, improve the mode of cellular uptake, change the permeability through physiological barriers, and modify the rate of clearance through the body. To achieve either a targeting or sustained-release effect, water-soluble polymers have been synthesized that contain drug moieties as terminal groups, as part of the backbone, or as pendent groups on the polymer chain.

Representative compositions of the present invention include thyroid hormone or analogs thereof conjugated to polymers. Conjugation with polymers can be either through covalent or non-covalent linkages. In preferred embodiments, the polymer conjugation can occur through an ester linkage or an anhydride linkage. An example of a polymer conjugation through an ester linkage using polyvinyl alcohol is shown in FIG. **17**. In this preparation commercially available polyvinyl alcohol (or related co-polymers) can be esterified by treatment with the acid chloride of thyroid hormone analogs, including the acid chloride form. The hydrochloride salt is neutralized by the addition of triethylamine to afford triethylamine hydrochloride which can be washed away with water upon precipitation of the thyroid hormone ester polymer form for different analogs. The ester linkage to the polymer may undergo hydrolysis in vivo to release the active pro-angiogenesis thyroid hormone analog.

An example of a polymer conjugation through an anhydride linkage using acrylic acid ethylene co-polymer is shown in FIG. **18**. This is similar to the previous polymer covalent conjugation, however, this time it is through an anhydride linkage that is derived from reaction of an acrylic acid co-polymer. This anhydride linkage is also susceptible to hydrolysis in vivo to release thyroid hormone analog. Neutralization of the hydrochloric acid is accomplished by treatment with triethylamine and subsequent washing of the precipitated polyanhydride polymer with water removes the triethylamine hydrochloride byproduct. This reaction will lead to the formation of Thyroid hormone analog acrylic acid co-polymer+triethylamine. Upon in vivo hydrolysis, the thyroid hormone analog will be released over time that can be controlled plus acrylic acid ethylene Co-polymer.

Another representative polymer conjugation includes thyroid hormone or its analogs conjugated to polyethylene glycol (PEG). Attachment of PEG to various drugs, proteins and liposomes has been shown to improve residence time and decrease toxicity. PEG can be coupled to active agents through the hydroxyl groups at the ends of the chains and via other chemical methods. Peg itself, however, is limited to two active agents per molecule. In a different approach, copolymers of PEG and amino acids were explored as novel biomaterials which would retain the biocompatibility properties of PEG, but which would have the added advantage of numerous attachment points per molecule and which could be synthetically designed to suit a variety of applications.

Another representative polymer conjugation includes thyroid hormone or its analogs in non-covalent conjugation with polymers. This is shown in detail in FIG. **19**. A preferred non-covalent conjugation is entrapment of thyroid hormone or analogs thereof in a polylactic acid polymer. Polylactic acid polyester polymers (PLA) undergo hydrolysis in vivo to the lactic acid monomer and this has been exploited as a vehicle for drug delivery systems in humans. Unlike the prior two covalent methods where the thyroid hormone analog is linked by a chemical bond to the polymer, this would be a non-covalent method that would encapsulate the thyroid hormone analog into PLA polymer beads. This reaction will lead to the formation of Thyroid hormone analog containing PLA beads in water. Filter and washing will result in the formation of thyroid hormone analog containing PLA beads, which upon in vivo hydrolysis will lead to the generation of controlled levels of thyroid hormone plus lactic acid.

Still further, compositions of the present invention include thyroid hormone analogs conjugated to retinols (e.g., retinoic acid (i.e., Vitamin A), which bind to the thyroid hormone binding protein transthyretin ("TTR") and retinoic binding protein ("RBP"). Thyroid hormone analogs can also be conjugated with halogenated stilbesterols, alone or in combination with retinoic acid, for use in detecting and suppressing amyloid plaque. These analogs combine the advantageous properties of T4-TTR, namely, their rapid uptake and prolonged retention in brain and amyloids, with the properties of halogen substituents, including certain useful halogen isotopes for PET imaging including fluorine-18, iodine-123, iodine-124, iodine-131, bromine-75, bromine-76, bromine-77 and bromine-82.

Furthermore, nanotechnology can be used for the creation of useful materials and structures sized at the nanometer scale. The main drawback with biologically active substances is fragility. Nanoscale materials can be combined with such biologically active substances to dramatically improve the durability of the substance, create localized high concentrations of the substance and reduce costs by minimizing losses. Therefore, additional polymeric conjugations include nano-

particle formulations of thyroid hormones and analogs thereof. In such an embodiment, nano-polymers and nanoparticles can be used as a matrix for local delivery of thyroid hormone and its analogs. This will aid in time controlled delivery into the cellular and tissue target.

Compositions of the present invention include both thyroid hormone, analogs, and derivatives either alone or in covalent or non-covalent conjugation with polymers. Examples of representative analogs and derivatives are shown in FIG. **20**, Tables A-D. Table A shows T2, T3, T4, and bromo-derivatives. Table B shows alanyl side chain modifications. Table C shows hydroxy groups, diphenyl ester linkages, and D-configurations. Table D shows tyrosine analogs.

The terms "anti-angiogenesis agent" or anti-angiogenic agent" refer to any compound or substance that inhibits or discourages angiogenesis, whether alone or in combination with another substance. Examples include, but are not limited to, TETRAC, TRIAC, XT 199, and mAb LM609.

The Role of Thyroid Hormone, Analogs, and Polymeric Conjugations in Promoting Angiogenesis

The pro-angiogenic effect of thyroid hormone analogs or polymeric forms depends upon a non-genomic initiation, as tested by the susceptibility of the hormonal effect to reduction by pharmacological inhibitors of the MAPK signal transduction pathway. Such results indicates that another consequence of activation of MAPK by thyroid hormone is new blood vessel growth. The latter is initiated nongenomically, but of course, requires a consequent complex gene transcription program. The ambient concentrations of thyroid hormone are relatively stable. The CAM model, at the time we tested it, was thyroprival and thus may be regarded as a system, which does not reproduce the intact organism.

The availability of a chick chorioallantoic membrane (CAM) assay for angiogenesis has provided a model in which to quantitate angiogenesis and to study possible mechanisms involved in the induction by thyroid hormone of new blood vessel growth. The present application discloses a pro-angiogenic effect of T_4 that approximates that in the CAM model of FGF2 and that can enhance the action of suboptimal doses of FGF2. It is further disclosed that the pro-angiogenic effect of the hormone is initiated at the plasma membrane and is dependent upon activation by T_4 of the MAPK signal transduction pathway. As provided above, methods for treatment of occlusive peripheral vascular disease and coronary diseases, in particular, the occlusion of coronary vessels, and disorders associated with the occlusion of the peripheral vasculature and/or coronary blood vessels are disclosed. Also disclosed are compositions and methods for promoting angiogenesis and/or recruiting collateral blood vessels in a patient in need thereof. The compositions include an effective amount of Thyroid hormone analogs, polymeric forms, and derivatives. The methods involve the co-administration of an effective amount of thyroid hormone analogs, polymeric forms, and derivatives in low, daily dosages for a week or more with other standard pro-angiogenesis growth factors, vasodilators, anticoagulants, thrombolytics or other vascular-related therapies.

The CAM assay has been used to validate angiogenic activity of a variety of growth factors and compounds believed to promote angiogenesis. For example, T_4 in physiological concentrations was shown to be pro-angiogenic in this in vitro model and on a molar basis to have the activity of FGF2. The presence of PTU did not reduce the effect of T_4, indicating that de-iodination of T_4 to generate T_3 was not a prerequisite in this model. A summary of the pro-angiogenesis effects of various thyroid hormone analogs is listed in Table 1.

TABLE 1

Pro-angiogenesis Effects of Various Thyroid Hormone Analogs in the CAM Model

TREATMENT	ANGIOGENESIS INDEX
PBS (Control)	89.4 ± 9.3
DITPA (0.01 uM)	133.0 ± 11.6
DITPA (0.1 uM)	167.3 ± 12.7
DITPA (0.2 mM)	117.9 ± 5.6
GC-1 (0.01 uM)	169.6 ± 11.6
GC-1 (0.1 uM)	152.7 ± 9.0
T4 agarose (0.1 uM)	195.5 + 8.5
T4 (0.1 uM)	143.8 ± 7.9
FGF2 (1 ug)	155 ± 9

n = 8 per group

The appearance of new blood vessel growth in this model requires several days, indicating that the effect of thyroid hormone was wholly dependent upon the interaction of the nuclear receptor for thyroid hormone (TR) with the hormone. Actions of iodothyronines that require intranuclear complexing of TR with its natural ligand, T_3, are by definition, genomic, and culminate in gene expression. On the other hand, the preferential response of this model system to T_4-rather than T_3, the natural ligand of TR-raised the possibility that angiogenesis might be initiated nongenomically at the plasma membrane by T_4 and culminate in effects that require gene transcription. Non-genomic actions of T_4 have been widely described, are usually initiated at the plasma membrane and may be mediated by signal transduction pathways. They do not require intranuclear ligand of iodothyronine and TR, but may interface with or modulate gene transcription. Non-genomic actions of steroids have also been well described and are known to interface with genomic actions of steroids or of other compounds. Experiments carried out with T_4 and tetrac or with agarose-T_4 indicated that the pro-angiogenic effect of T_4 indeed very likely was initiated at the plasma membrane. Tetrac blocks membrane-initiated effects of T_4, but does not, itself, activate signal transduction. Thus, it is a probe for non-genomic actions of thyroid hormone. Agarose-T_4 is thought not to gain entry to the cell interior and has been used to examine models for possible cell surface-initiated actions of the hormone. Investigations of the pro-angiogenic effects of thyroid hormone in the chick chorioallantoic membrane ("CAM") model demonstrate that generation of new blood vessels from existing vessels was promoted two- to three-fold by either L-thyroxine (T_4) or 3,5,3'-triiodo-L-thyronine (T_3) at 10^{-7}-10^{-9} M. More interestingly, T_4-agarose, a thyroid hormone analog that does not cross the cell membrane, produced a potent pro-angiogenesis effect comparable to that obtained with T_3 or T_4.

In part, this invention provides compositions and methods for promoting angiogenesis in a subject in need thereof. Conditions amenable to treatment by promoting angiogenesis include, for example, occlusive peripheral vascular disease and coronary diseases, in particular, the occlusion of coronary vessels, and disorders associated with the occlusion of the peripheral vasculature and/or coronary blood vessels, erectile dysfunction, stroke, and wounds. Also disclosed are compositions and methods for promoting angiogenesis and/or recruiting collateral blood vessels in a patient in need thereof. The compositions include an effective amount of polymeric forms of thyroid hormone analogs and derivatives and an effective amount of an adenosine and/or nitric oxide donor. The compositions can be in the form of a sterile, injectable, pharmaceutical formulation that includes an angiogenically effective amount of thyroid hormone-like substance and

adenosine derivatives in a physiologically and pharmaceutically acceptable carrier, optionally with one or more excipients.

Myocardial Infarction

A major reason for heart failure following acute myocardial infarction is an inadequate response of new blood vessel formation, i.e., angiogenesis. Thyroid hormone and its analogs are beneficial in heart failure and stimulate coronary angiogenesis. The methods of the invention include, in part, delivering a single treatment of a thyroid hormone analog at the time of infarction either by direct injection into the myocardium, or by simulation of coronary injection by intermittent aortic ligation to produce transient isovolumic contractions to achieve angiogenesis and/or ventricular remodeling.

Accordingly, in one aspect the invention features methods for treating occlusive vascular disease, coronary disease, myocardial infarction, ischemia, stroke, and/or peripheral artery vascular disorders by promoting angiogenesis by administering to a subject in need thereof an amount of a polymeric form of thyroid hormone, or an analog thereof, effective for promoting angiogenesis.

Examples of polymeric forms of thyroid hormone analogs are also provided herein and can include triiodothyronine (T3), levothyroxine (T4), (GC-1), or 3,5-diiodothyropropionic acid (DITPA) conjugated to polyvinyl alcohol, acrylic acid ethylene co-polymer, polylactic acid, or agarose.

The methods also involve the co-administration of an effective amount of thyroid hormone-like substance and an effective amount of an adenosine and/or NO donor in low, daily dosages for a week or more. One or both components can be delivered locally via catheter. Thyroid hormone analogs, and derivatives in vivo can be delivered to capillary beds surrounding ischemic tissue by incorporation of the compounds in an appropriately sized liposome or microparticle. Thyroid hormone analogs, polymeric forms and derivatives can be targeted to ischemic tissue by covalent linkage with a suitable antibody.

The method may be used as a treatment to restore cardiac function after a myocardial infarction. The method may also be used to improve blood flow in patients with coronary artery disease suffering from myocardial ischemia or inadequate blood flow to areas other than the heart including, for example, occlusive peripheral vascular disease (also known as peripheral arterial occlusive disease), or erectile dysfunction.

Wound Healing

Wound angiogenesis is an important part of the proliferative phase of healing. Healing of any skin wound other than the most superficial cannot occur without angiogenesis. Not only does any damaged vasculature need to be repaired, but the increased local cell activity necessary for healing requires an increased supply of nutrients from the bloodstream. Moreover, the endothelial cells which form the lining of the blood vessels are important in themselves as organizers and regulators of healing.

Thus, angiogenesis provides a new microcirculation to support the healing wound. The new blood vessels become clinically visible within the wound space by four days after injury. Vascular endothelial cells, fibroblasts, and smooth muscle cells all proliferate in coordination to support wound granulation. Simultaneously, re-epithelialization occurs to reestablish the epithelial cover. Epithelial cells from the wound margin or from deep hair follicles migrate across the wound and establish themselves over the granulation tissue and provisional matrix. Growth factors such as keratinocyte growth factor (KGF) mediate this process. Several models (sliding versus rolling cells) of epithelialization exist.

As thyroid hormones regulate metabolic rate, when the metabolism slows down due to hypothyroidism, wound healing also slows down. The role of topically applied thyroid hormone analogs or polymeric forms in wound healing therefore represents a novel strategy to accelerate wound healing in diabetics and in non-diabetics with impaired wound healing abilities. Topical administration can be in the form of attachment to a band-aid. Additionally, nano-polymers and nano-particles can be used as a matrix for local delivery of thyroid hormone and its analogs. This will aid in time-controlled delivery into the cellular and tissue target.

Accordingly, another embodiment of the invention features methods for treating wounds by promoting angiogenesis by administering to a subject in need thereof an amount of a polymeric form of thyroid hormone, or an analog thereof, effective for promoting angiogenesis. For details, see Examples 9A and 9B.

The Role of Thyroid Hormone, Analogs, and Polymeric Conjugations in Combination with Nerve Growth Factors in Inducing and Maintaining Neuronal Cells

Contrary to traditional understanding of neural induction, the present invention is partly based on the unexpected finding that mechanisms that initiate and maintain angiogenesis are effective promoters and sustainers of neurogenesis. These methods and compositions are useful, for example, for the treatment of motor neuron injury and neuropathy in trauma, injury and neuronal disorders. This invention discloses the use of various pro-angiogenesis strategies alone or in combination with nerve growth factor or other neurogenesis factors. Pro-angiogenesis factors include polymeric thyroid hormone analogs as illustrated herein. The polymeric thyroid hormone analogs and its polymeric conjugates alone or in combination with other pro-angiogenesis growth factors known in the art and with nerve growth factors or other neurogenesis factors can be combined for optimal neurogenesis.

Disclosed are therapeutic treatment methods, compositions and devices for maintaining neural pathways in a mammal, including enhancing survival of neurons at risk of dying, inducing cellular repair of damaged neurons and neural pathways, and stimulating neurons to maintain their differentiated phenotype. Additionally, a composition containing polymeric thyroid hormone analogs, and combinations thereof, in the presence of anti-oxidants and/or anti-inflammatory agents demonstrate neuronal regeneration and protection.

The present invention also provides thyroid hormones, analogs, and polymeric conjugations, alone or in combination with nerve growth factors or other neurogenesis factors, to enhance survival of neurons and maintain neural pathways. As described herein, polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors are capable of enhancing survival of neurons, stimulating neuronal CAM expression, maintaining the phenotypic expression of differentiated neurons, inducing the redifferentiation of transformed cells of neural origin, and stimulating axonal growth over breaks in neural processes, particularly large gaps in axons. Morphogens also protect against tissue destruction associated with immunologically-related nerve tissue damage. Finally, polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors may be used as part of a method for monitoring the viability of nerve tissue in a mammal.

The present invention also provides effects of polymeric thyroid hormones on synapse formation between cultured rat cortical neurons, using a system to estimate functional synapse formation in vitro. Exposure to 10-9 M polymeric thyroid hormones, 3,5,3'-triiodothyronine or thyroxine, caused

an increase in the frequency of spontaneous synchronous oscillatory changes in intracellular calcium concentration, which correlated with the number of synapses formed. The detection of synaptic vesicle-associated protein synapsin I by immunocytochemical and immunoblot analysis also confirmed that exposure to thyroxine facilitated synapse formation. The presence of amiodarone, an inhibitor of 5'-deiodinase, or amitrole, a herbicide, inhibited the synapse formation in the presence of thyroxine. Thus, the present invention also provides a useful in vitro assay system for screening of miscellaneous chemicals that might interfere with synapse formation in the developing CNS by disrupting the polymeric thyroid system.

As a general matter, methods of the present invention may be applied to the treatment of any mammalian subject at risk of or afflicted with a neural tissue insult or neuropathy. The invention is suitable for the treatment of any primate, preferably a higher primate such as a human. In addition, however, the invention may be employed in the treatment of domesticated mammals which are maintained as human companions (e.g., dogs, cats, horses), which have significant commercial value (e.g., goats, pigs, sheep, cattle, sporting or draft animals), which have significant scientific value (e.g., captive or free specimens of endangered species, or inbred or engineered animal strains), or which otherwise have value.

The polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors described herein enhance cell survival, particularly of neuronal cells at risk of dying. For example, fully differentiated neurons are non-mitotic and die in vitro when cultured under standard mammalian cell culture conditions, using a chemically defined or low serum medium known in the art. See, for example, Chamess, J. Biol. Chem. 26: 3164-3169 (1986) and Freese, et al., Brain Res. 521: 254-264 (1990). However, if a primary culture of non-mitotic neuronal cells is treated with polymeric thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors, the survival of these cells is enhanced significantly. For example, a primary culture of striatal basal ganglia isolated from the substantia nigra of adult rat brain was prepared using standard procedures, e.g., by dissociation by trituration with pasteur pipette of substantia nigra tissue, using standard tissue culturing protocols, and grown in a low serum medium, e.g., containing 50% DMEM (Dulbecco's modified Eagle's medium), 50% F-12 medium, heat inactivated horse serum supplemented with penicillin/streptomycin and 4 g/l glucose. Under standard culture conditions, these cells are undergoing significant cell death by three weeks when cultured in a serum-free medium. Cell death is evidenced morphologically by the inability of cells to remain adherent and by changes in their ultrastmctural characteristics, e.g., by chromatin clumping and organelle disintegration. Specifically, cells remained adherent and continued to maintain the morphology of viable differentiated neurons. In the absence of thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors treatment, the majority of the cultured cells dissociated and underwent cell necrosis.

Dysfunctions in the basal ganglia of the substantia nigra are associated with Huntington's chorea and parkinsonism in vivo. The ability of the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors defined herein to enhance neuron survival indicates that these polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors will be useful as part of a therapy to enhance survival of neuronal cells at risk of dying in vivo due, for example, to a neuropathy or chemical or mechanical trauma. The present invention further provides that these polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors provide a useful therapeutic agent to treat neuropathies which affect the striatal basal ganglia, including Huntington's chorea and Parkinson's disease. For clinical applications, the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors may be administered or, alternatively, a polymeric thyroid hormone analog alone or in combination with nerve growth factors or other neurogenesis factors-stimulating agent may be administered.

The thyroid hormone compounds described herein can also be used for nerve tissue protection from chemical trauma. The ability of the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors described herein to enhance survival of neuronal cells and to induce cell aggregation and cell—cell adhesion in redifferentiated cells, indicates that the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors will be useful as therapeutic agents to maintain neural pathways by protecting the cells defining the pathway from the damage caused by chemical trauma. In particular, the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors can protect neurons, including developing neurons, from the effects of toxins known to inhibit the proliferation and migration of neurons and to interfere with cell—cell adhesion. Examples of such toxins include ethanol, one or more of the toxins present in cigarette smoke, and a variety of opiates. The toxic effects of ethanol on developing neurons induces the neurological damage manifested in fetal alcohol syndrome. The polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors also may protect neurons from the cytotoxic effects associated with excitatory amino acids such as glutamate.

For example, ethanol inhibits the cell—cell adhesion effects induced in polymeric thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors-treated NG108-15 cells when provided to these cells at a concentration of 25-50 mM. Half maximal inhibition can be achieved with 5-10 mM ethanol, the concentration of blood alcohol in an adult following ingestion of a single alcoholic beverage. Ethanol likely interferes with the homophilic binding of CAMs between cells, rather than their induction, as polymeric thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors-induced N-CAM levels are unaffected by ethanol. Moreover, the inhibitory effect is inversely proportional to polymeric thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors concentration. Accordingly, it is envisioned that administration of a polymeric thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors or polymeric thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors-stimulating agent to neurons, particularly developing neurons, at risk of damage from exposure to toxins such as ethanol, may protect these cells from nerve tissue damage by overcoming the toxin's inhibitory effects. The polymeric thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors described herein also are useful in therapies to treat damaged neural pathways resulting from a neuropathy induced by exposure to these toxins.

The in vivo activities of the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors described herein also are assessed

readily in an animal model as described herein. A suitable animal, preferably exhibiting nerve tissue damage, for example, genetically or environmentally induced, is injected intracerebrally with an effective amount of a polymeric thyroid hormone analogs alone or in combination with nerve growth factor or other neurogenesis factors in a suitable therapeutic formulation, such as phosphate-buffered saline, pH 7. The polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors preferably is injected within the area of the affected neurons. The affected tissue is excised at a subsequent time point and the tissue evaluated morphologically and/or by evaluation of an appropriate biochemical marker (e.g., by polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors or N-CAM localization; or by measuring the dose-dependent effect on a biochemical marker for CNS neurotrophic activity or for CNS tissue damage, using for example, glial fibrillary acidic protein as the marker. The dosage and incubation time will vary with the animal to be tested. Suitable dosage ranges for different species may be determined by comparison with established animal models. Presented below is an exemplary protocol for a rat brain stab model.

Briefly, male Long Evans rats, obtained from standard commercial sources, are anesthetized and the head area prepared for surgery. The calvariae is exposed using standard surgical procedures and a hole drilled toward the center of each lobe using a 0.035K wire, just piercing the calvariae. 25 ml solutions containing either polymeric thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors (e.g., OP-1, 25 mg) or PBS then is provided to each of the holes by Hamilton syringe. Solutions are delivered to a depth approximately 3 mm below the surface, into the underlying cortex, corpus callosum and hippocampus. The skin then is sutured and the animal allowed to recover.

Three days post surgery, rats are sacrificed by decapitation and their brains processed for sectioning. Scar tissue formation is evaluated by immunofluorescence staining for glial fibrillary acidic protein, a marker protein for glial scarring, to qualitatively determine the degree of scar formation. Glial fibrillary acidic protein antibodies are available commercially, e.g., from Sigma Chemical Co., St. Louis, Mo. Sections also are probed with anti-OP-1 antibodies to determine the presence of OP-1. Reduced levels of glial fibrillary acidic protein are anticipated in the tissue sections of animals treated with the polymeric thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors, evidencing the ability of polymeric thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors to inhibit glial scar formation and stimulate nerve regeneration.

The Role of Thyroid Hormone, Analogs, and Polymeric Conjugations for Brain Imaging, Diagnosis, and Therapies of Neurodegenerative Diseases

The present invention relates to novel pharmaceutical and radiopharmaceuticals useful for the early diagnosis, prevention, and treatment of neurodegenerative disease, such as, for example, Alzheimer's disease. The invention also includes novel chemical compounds having specific binding in a biological system and capable of being used for positron emission tomography (PET), single photon emission (SPECT) imaging methods, and magnetic resonance (MRI) imaging methods. The ability of T4 and other thyroid hormone analogs to bind to localized ligands within the body makes it possible to utilize such compounds for in situ imaging of the ligands by PET, SPECT, MRI, and similar imaging methods. In principle, nothing need be known about the nature of the ligand, as long as binding occurs, and such binding is specific for a class of cells, organs, tissues or receptors of interest.

PET imaging is accomplished with the aid of tracer compounds labeled with a positron-emitting isotope (Goodman, M. M. Clinical Positron Emission Tomography, Mosby Yearbook, 1992, K. F. Hubner et al., Chapter 14). For most biological materials, suitable isotopes are few. The carbon isotope, ^{11}C, has been used for PET, but its short half-life of 20.5 minutes limits its usefulness to compounds that can be synthesized and purified quickly, and to facilities that are proximate to a cyclotron where the precursor C^{11} starting material is generated. Other isotopes have even shorter half-lives. N^{13} has a half-life of 10 minutes and O^{15} has an even shorter half-life of 2 minutes. The emissions of both are more energetic than those of C^{11}. Nevertheless, PET studies have been carried out with these isotopes (Hubner, K. F., in Clinical Positron Emission Tomography, Mosby Year Book, 1992, K. F. Hubner, et al., Chapter 2). A more useful isotope, ^{18}F, has a half-life of 110 minutes. This allows sufficient time for incorporation into a radio-labeled tracer, for purification and for administration into a human or animal subject. In addition, facilities more remote from a cyclotron, up to about a 200 mile radius, can make use of F^{18} labeled compounds. Disadvantages of ^{18}F are the relative scarcity of fluorinated analogs that have functional equivalence to naturally-occurring biological materials, and the difficulty of designing methods of synthesis that efficiently utilize the starting material generated in the cyclotron. Such starting material can be either fluoride ion or fluorine gas. In the latter case only one fluorine atom of the bimolecular gas is actually a radionuclide, so the gas is designated F-F^{18}. Reactions using F-F^{18} as starting material therefore yield products having only one half the radionuclide abundance of reactions utilizing K. F^{18} as starting material. On the other hand, F^{18} can be prepared in curie quantities as fluoride ion for incorporation into a radiopharmaceutical compound in high specific activity, theoretically 1.7 Ci/nmol using carrier-free nucleophilic substitution reactions. The energy emission of F^{18} is 0.635 MeV, resulting in a relatively short, 2.4 mm average positron range in tissue, permitting high resolution PET images.

SPECT imaging employs isotope tracers that emit high energy photons (.gamma.-emitters). The range of useful isotopes is greater than for PET, but SPECT provides lower three-dimensional resolution. Nevertheless, SPECT is widely used to obtain clinically significant information about analog binding, localization and clearance rates. A useful isotope for SPECT imaging is I^{123} α-gamma.-emitter with a 13.3 hour half life. Compounds labeled with I^{123} can be shipped up to about 1000 miles from the manufacturing site, or the isotope itself can be transported for on-site synthesis. Eighty-five percent of the isotope's emissions are 159 KeV photons, which is readily measured by SPECT instrumentation currently in use. The compounds of the invention can be labeled with Technetium. Technetium-99m is known to be a useful radionuclide for SPECT imaging. The T4 analogs of the invention are joined to a Tc-99m metal cluster through a 4-6 carbon chain which can be saturated or possess a double or triple bond.

Use of F^{18} labeled compounds in PET has been limited to a few analog compounds. Most notably, ^{18}F-fluorodeoxyglucose has been widely used in studies of glucose metabolism and localization of glucose uptake associated with brain activity. ^{18}F-L-fluorodopa and other dopamine receptor analogs have also been used in mapping dopamine receptor distribution.

Other halogen isotopes can serve for PET or SPECT imaging, or for conventional tracer labeling. These include ^{75}Br,

^{76}Br, ^{77}Br and ^{82}Br as having usable half-lives and emission characteristics. In general, the chemical means exist to substitute any halogen moiety for the described isotopes. Therefore, the biochemical or physiological activities of any halogenated homolog of the described compounds are now available for use by those skilled in the art, including stable isotope halogen homolog. Astatine can be substituted for other halogen isotypes. ^{210}At, for example, emits alpha particles with a half-life of 8.3 h. Other isotopes also emit alpha particles with reasonably useful half-lives. At-substituted compounds are therefore useful for brain therapy, where binding is sufficiently brain-specific.

Numerous studies have demonstrated increased incorporation of carbohydrates and amino acids into malignant brain cells. This accumulation is associated with accelerated proliferation and protein synthesis of such cells. The glucose analog ^{18}F-2-fluoro-2-deoxy-D-glucose (2-FDG) has been used for distinguishing highly malignant brain brains from normal brain tissue or benign growths (DiChiro, G. et al. (1982) Neurology (NY) 32:1323-1329. However, fluorine-18 labeled 2-FDG is not the agent of choice for detecting low grade brain brains because high uptake in normal tissue can mask the presence of a brain. In addition, fluorine-18 labeled 2-FDG is not the ideal radiopharmaceutical for distinguishing lung brains from infectious tissue or detecting ovarian carcinoma because of high uptake of the 2-FDG radioactivity in infectious tissue and in the bladder, respectively. The naturally occurring amino acid methionine, labeled with carbon-11, has also been used to distinguish malignant tissue from normal tissue. But it too has relatively high uptake in normal tissue. Moreover, the half-life of carbon-11 is only 20 minutes; therefore C11 methionine can not be stored for a long period of time.

Cerebrospinal fluid ("CSF") transthyretin ("TTR"), the main CSF thyroxine (T4) carrier protein in the rat and the human is synthesized in the choroid plexus ("CP"). After injection of ^{125}I-T4 in the rat, radioactive T4 accumulates first in the CP, then in the CSF and later in the brain (Chanoine J P, Bravennan L E. The role of transthyretin in the transport of thyroid hormone to cerebrospinal fluid and brain. Acta Med. Austriaca. 1992; 19 Suppl 1:25-8).

Compounds of the invention provide substantially improved PET imaging for areas of the body having amyloid protein, especially of the brain. All the available positron-emitting isotopes which could be incorporated into a biologically-active compound have short half-lives. The practical utility of such labeled compounds is therefore dependent on how rapidly the labeled compound can be synthesized, the synthetic yield and the radiochemical purity of the final product. Even the shipping time from the isotope source, a cyclotron facility, to the hospital or laboratory where PET imaging is to take place, is limited. A rough calculation of the useful distance is about two miles per minute of half-life. Thus C^{11}, with a half-life of 20.5 m is restricted to about a 40 mile radius from a source whereas compounds labeled with F^{18} can be used within about a 200 mile radius. Further requirements of an ^{18}F-labeled compound are that it have the binding specificity for the receptor or target molecule it is intended to bind, that non-specific binding to other targets be sufficiently low to permit distinguishing between target and non-target binding, and that the label be stable under conditions of the test to avoid exchange with other substances in the test environment. More particularly, compounds of the invention must display adequate binding to the desired target while failing to bind to any comparable degree with other tissues or cells.

A partial solution to the stringent requirements for PET imaging is to employ .gamma-emitting isotopes in SPECT imaging. I^{123} is a commonly used isotopic marker for SPECT, having a half-life of 13 hours for a useful range of over 1000 miles from the site of synthesis. Compounds of the invention can be rapidly and efficiently labeled with I^{123} for use in SPECT analysis as an alternative to PET imaging. Furthermore, because of the fact that the same compound can be labeled with either isotope, it is possible for the first time to compare the results obtained by PET and SPECT using the same tracer.

The specificity of brain binding also provides utility for I-substituted compounds of the invention. Such compounds can be labeled with short-lived ^{123}I for SPECT imaging or with longer-lived ^{125}I for longer-term studies such as monitoring a course of therapy. Other iodine and bromine isotopes can be substituted for those exemplified.

The compounds of the invention therefore provide improved methods for brain imaging using PET and SPECT. The methods entail administering to a subject (which can be human or animal, for experimental and/or diagnostic purposes) an image-generating amount of a compound of the invention, labeled with the appropriate isotope and then measuring the distribution of the compound by PET if F^{18} or other positron emitter is employed, or SPECT if I^{123} or other gamma emitter is employed. An image-generating amount is that amount which is at least able to provide an image in a PET or SPECT scanner, taking into account the scanner's detection sensitivity and noise level, the age of the isotope, the body size of the subject and route of administration, all such variables being exemplary of those known and accounted for by calculations and measurements known to those skilled in the art without resort to undue experimentation.

It will be understood that compounds of the invention can be labeled with an isotope of any atom or combination of atoms in the structure. While F^{18}, I^{123}, and I^{125} have been emphasized herein as being particularly useful for PET, SPECT and tracer analysis, other uses are contemplated including those flowing from physiological or pharmacological properties of stable isotope homolog and will be apparent to those skilled in the art.

The invention also provides for technetium (Tc) labeling via Tc adducts. Isotopes of Tc, notably Tc^{99m}, have been used for brain imaging. The present invention provides Tc-complexed adducts of compounds of the invention, which are useful for brain imaging. The adducts are Tc-coordination complexes joined to the cyclic amino acid by a 4-6 carbon chain which can be saturated or possess a double or triple bond. Where a double bond is present, either E (trans) or Z (cis) isomers can be synthesized, and either isomer can be employed. Synthesis is described for incorporating the ^{99m}Tc isotope as a last step, to maximize the useful life of the isotope.

The following methods were employed in procedures reported herein. ^{18}F-Fluoride was produced from a Seimens cyclotron using the $^{18}O(p,n)\ ^{18}F$ reaction with 11 MeV protons on 95% enriched ^{18}O water. All solvents and chemicals were analytical grade and were used without further purification. Melting points of compounds were determined in capillary tubes by using a Buchi SP apparatus. Thin-layer chromatographic analysis (TLC) was performed by using 250-mm thick layers of silica gel G PF-254 coated on aluminum (obtained from Analtech, Inc.). Column chromatography was performed by using 60-200 mesh silica gel (Aldrich Co.). Infrared spectra (IR) were recorded on a Beckman 18A spectrophotometer with NaCl plates. Proton nuclear magnetic resonance spectra (1H NMR) were obtained at 300 MHz with a Nicolet high-resolution instrument.

In another aspect, the invention is directed to a method of using a compound of the invention for the manufacture of a radiopharmaceutical for the diagnosis of Alzheimer's disease in a human. In another aspect, the invention is directed to a method of preparing compounds of the invention.

The compounds of the invention as described herein are the thyroid hormone analogs or other TTR binding ligands, which bind to TTR and have the ability to pass the blood-brain barrier. The compounds are therefore suited as in vivo diagnostic agents for imaging of Alzheimer's disease. The detection of radioactivity is performed according to well-known procedures in the art, either by using a gamma camera or by positron emission tomography (PET).

Preferably, the free base or a pharmaceutically acceptable salt form, e.g. a monochloride or dichloride salt, of a compound of the invention is used in a galenical formulation as diagnostic agent. The galenical formulation containing the compound of the invention optionally contains adjuvants known in the art, e.g. buffers, sodium chloride, lactic acid, surfactants etc. A sterilization by filtration of the galenical formulation under sterile conditions prior to usage is possible.

The radioactive dose should be in the range of 1 to 100 mCi, preferably 5 to 30 mCi, and most preferably 5 to 20 mCi per application.

Of the various aspects of the invention, certain compounds (for example, compounds disclosed in Examples 16-20) are preferred. Especially preferred are such compounds for use as diagnostic agents in positron emission tomography (PET).

The compounds of the present invention may be administered by any suitable route, preferably in the form of a pharmaceutical composition adapted to such a route, and in a dose effective to bind TTR in the brain and thereby be detected by gamma camera or PET. Typically, the administration is parenteral, e.g., intravenously, intraperitoneally, subcutaneously, intradermally, or intramuscularly. Intravenous administration is preferred. Thus, for example, the invention provides compositions for parenteral administration which comprise a solution of contrast media dissolved or suspended in an acceptable carrier, e.g., serum or physiological sodium chloride solution.

Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyl oleate. Other pharmaceutically acceptable carriers, non-toxic excipients, including salts, preservatives, buffers and the like, are described, for instance, in REMMINGTON'S PHARMACEUTICAL SCIENCES, 15.sup.th Ed. Easton: Mack Publishing Co., pp. 1405-1412 and 1461-1487 (1975) and THE NATIONAL FORMULARY XIV., 14.sup.th Ed. Washington: American Pharmaceutical Association (1975). Aqueous carriers, are preferred.

Pharmaceutical composition of this invention are produced in a manner known per se by suspending or dissolving the compounds of this invention—optionally combined with the additives customary in galenic pharmacy—in an aqueous medium and then optionally sterilizing the suspension or solution. Suitable additives are, for example, physiologically acceptable buffers (such as, for instance, tromethamine), additions of complexing agents (e.g., diethylenetriaminepentaacetic acid) or—if required—electrolytes, e.g., sodium chloride or—if necessary—antioxidants, such as ascorbic acid, for example.

If suspensions or solutions of the compounds of this invention in water or physiological saline solution are desirable for enteral administration or other purposes, they are mixed with one or several of the auxiliary agents (e.g., methylcellulose, lactose, mannitol) and/or tensides (e.g., lecithins, "Tween", "Myrj") and/or flavoring agents to improve taste (e.g., ethereal oils), as customary in galenic pharmacy.

The compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

For the compounds according to the invention having radioactive halogens, these compounds can be shipped as "hot" compounds, i.e., with the radioactive halogen in the compound and administered in e.g., a physiologically acceptable saline solution. In the case of the metal complexes, these compounds can be shipped as "cold" compounds, i.e., without the radioactive ion, and then mixed with Tc-generator eluate or Re-generator eluate.

The Role of Thyroid Hormone Analogs and Polymeric Conjugations in Modulating the Actions of Polypeptides Whose Cell Surface Receptors are Clustered Around Integrin αβ3, or Other RGD-Containing Compounds

Integrin αVβ3 is a heterodimeric plasma membrane protein with several extracellular matrix protein ligands containing an amino acid sequence Arg-Gly-Asp ("RGD"). Using purified integrin, we discovered that integrin αVβ3 binds T4 and that this interaction is perturbed by αVβ3 antagonists. Radioligand-binding studies revealed that purified αVβ3 binds T4 with high affinity (EC50, 371 pM), and appears to bind T4 preferentially over T3. This is consistent with previous reports that show MAPK activation and nuclear translocation, as well as hormone-induced angiogenesis, by T4, compared to T3. Integrin αVβ3 antagonists inhibit binding of T4 to the integrin and, importantly, prevent activation by T4 of the MAPK signaling cascade. This functional consequence-MAPK activation—of hormone-binding to the integrin, together with inhibition of the MAPK-dependent proangiogenic action of thyroid hormone by integrin αVβ3 antagonists, allow us to describe the iodothyronine-binding site on the integrin as a receptor. It should be noted that 3-iodothyronamine, a thyroid hormone derivative, has recently been shown by Scanlan et al. to bind to a trace amine receptor (TAR I), but the actions of this analog interestingly are antithetic to those of T4 and T3.

The traditional ligands of integrins are proteins. That a small molecule, thyroid hormone, is also a ligand of an integrin is a novel finding. The present invention also discloses that, resveratrol, a polyphenol with some estrogenic activity, binds to integrin αVβ3 with a functional cellular consequence, apoptosis, different from those that result from the binding of thyroid hormone. The site on the integrin at which T4 binds is at or near the RGD binding groove of the heterodimeric integrin. It is possible, however, that αVβ3 binds T4 elsewhere on the protein and that the occupation of the RGD recognition site by tetrac or by RGD-containing peptides allosterically blocks the T4 binding site or causes a conformational change within the integrin that renders the T4 site unavailable. Accordingly, the modulation by T4 of the laminin-integrin interaction of astrocytes may be a consequence of binding of the hormone to the integrin. The possibility thus exists that at the cell exterior thyroid hormone may

affect the liganding by integrin αVβ3 of extracellular matrix proteins in addition to laminin.

Actions of T4 that are nongenomic in mechanism have been well documented in recent years. A number of these activities are MAPK-mediated. We have shown that initial steps in activation of the MAPK cascade by thyroid hormone, including activation of protein kinase C, are sensitive to GTPγS and pertussis toxin, indicating that the plasma membrane receptor for thyroid hormone is G protein-sensitive. It should be noted that certain cellular functions mediated by integrin αVβ3 have been shown by others to be G protein-modulated. For example, site-directed mutagenesis of the RGD binding domain abolishes the ability of the nucleotide receptor P2Y2 to activate G_0, while the activation of G_q, was not affected. Wang et al. demonstrated that an integrin-associated protein, IAP/CD47, induced smooth muscle cell migration via G_i-mediated inhibition of MAPK activation.

In addition to linking the binding of T4 and other analogs by integrin αVβ3 to activation of a specific intracellular signal transduction pathway, the present invention also discloses that the liganding of the hormone by the integrin is critical to induction by T4 of MAPK-dependent angiogenesis. In the CAM model, significant vessel growth occurs after 48-72 h of T4 treatment, indicating that the plasma membrane effects of T4 can result in complex transcriptional changes. Thus, what is initiated as a nongenomic action of the hormone—transduction of the cell surface T4 signal—interfaces with genomic effects of the hormone that culminate in neovascularization. Interfaces of nongenomic and genomic actions of thyroid hormone have previously been described, e.g., MAPK-dependent phosphorylation at Ser-142 of TRβ1 that is initiated at the cell surface by T4 and that results in shedding by TR of corepressor proteins and recruitment of coactivators. The instant invention also discloses that T4 stimulates growth of C-6 glial cells by a MAPK-dependent mechanism that is inhibited by RGD peptide, and that thyroid hormone causes MAPK-mediated serine-phosphorylation of the nuclear estrogen receptor (ERα) in MCF-7 cells by a process we now know to be inhibitable by an RGD peptide. These findings in several cell lines all support the participation of the integrin in functional responses of cells to thyroid hormone.

Identification of αVβ3 as a membrane receptor for thyroid hormone indicates clinical significance of the interaction of the integrin and the hormone and the downstream consequence of angiogenesis. For example, αVβ3 is overexpressed in many tumors and this overexpression appears to play a role in tumor invasion and growth. Relatively constant circulating levels of thyroid hormone can facilitate tumor-associated angiogenesis. In addition to demonstrating the pro-angiogenic action of T4 in the CAM model here and elsewhere, the present invention also discloses that human dermal microvascular endothelial cells also form new blood vessels when exposed to thyroid hormone. Local delivery of αVβ3 antagonists or tetrac around tumor cells might inhibit thyroid hormone-stimulated angiogenesis. Although tetrac lacks many of the biologic activities of thyroid hormone, it does gain access to the interior of certain cells. Anchoring of tetrac, or specific RGD antagonists, to non-immunogenic substrates (agarose or polymers) would exclude the possibility that the compounds could cross the plasma membrane, yet retain as shown here the ability to prevent T4-induced angiogenesis. The agarose-T4 used in the present studies is thus a prototype for a new family of thyroid hormone analogues that have specific cellular effects, but do not gain access to the cell interior.

Accordingly, the Examples herein identify integrin αVβ3 as a cell surface receptor for thyroid hormone (L-thyroxine, T4) and as the initiation site for T4-induced activation of intracellular signaling cascades. αVβ3 dissociably binds radiolabeled T4 with high affinity; radioligand-binding is displaced by tetraiodothyroacetic acid (tetrac), αVβ3 antibodies and by an integrin RGD recognition site peptide. CV-1 cells lack nuclear thyroid hormone receptor but bear plasma membrane αVβ3; treatment of these cells with physiological concentrations of T4 activates the MAPK pathway, an effect inhibited by tetrac, RGD peptide and αVβ3 antibodies. Inhibitors of T4-binding to the integrin also block the MAPK-mediated pro-angiogenic action of T4. T4-induced phosphorylation of MAPK is blocked by siRNA knockdown of αV and β3. These findings indicate that T4 binds to αVβ3 near the RGD recognition site and show that hormone-binding to αVβ3 has physiologic consequences.

The Role of Thyroid Hormone Analogs, and Polymeric Conjugations in Inhibiting Angiogenesis

The invention also provides, in another part, compositions and methods for inhibiting angiogenesis in a subject in need thereof. Conditions amenable to treatment by inhibiting angiogenesis include, for example, primary or metastatic tumors and diabetic retinopathy. The compositions can include an effective amount of TETRAC, TRIAC or mAb LM609. The compositions can be in the form of a sterile, injectable, pharmaceutical formulation that includes an anti-angiogenically effective amount of an anti-angiogenic substance in a physiologically and pharmaceutically acceptable carrier, optionally with one or more excipients.

In a further aspect, the invention provides methods for treating a condition amenable to treatment by inhibiting angiogenesis by administering to a subject in need thereof an amount of an anti-angiogenesis agent effective for inhibiting angiogenesis.

Examples of the anti-angiogenesis agents used for inhibiting angiogenesis are also provided by the invention and include, but are not limited to, tetraiodothyroacetic acid (TETRAC), triiodothyroacetic acid (TRIAC), monoclonal antibody LM609, or combinations thereof. Such anti-angiogenesis agents can act at the cell surface to inhibit the pro-angiogenesis agents.

Cancer-Related New Blood Vessel Growth

Examples of the conditions amenable to treatment by inhibiting angiogenesis include, but are not limited to, primary or metastatic tumors, including, but not limited to glioma and breast cancer. In such a method, compounds which inhibit the thyroid hormone-induced angiogenic effect are used to inhibit angiogenesis. Details of such a method is illustrated in Example 12.

Diabetic Retinopathy

Examples of the conditions amenable to treatment by inhibiting angiogenesis include, but are not limited to diabetic retinopathy, and related conditions. In such a method, compounds which inhibit the thyroid hormone-induced angiogenic effect are used to inhibit angiogenesis. Details of such a method is illustrated in Examples 8A and B.

It is known that proliferative retinopathy induced by hypoxia (rather than diabetes) depends upon alphaV (αV) integrin expression (E Chavakis et al., Diabetologia 45:262-267, 2002). It is proposed herein that thyroid hormone action on a specific integrin alphaVbeta-3 (αVβ3) is permissive in the development of diabetic retinopathy. Integrin αVβ3 is identified herein as the cell surface receptor for thyroid hormone. Thyroid hormone, its analogs, and polymer conjugations, act via this receptor to induce angiogenesis.

Methods of Treatment and Formulations

Thyroid hormone analogs, polymeric forms, and derivatives can be used in a method for promoting angiogenesis in a patient in need thereof. The method involves the co-administration of an effective amount of thyroid hormone analogs, polymeric forms, and derivatives in low, daily dosages for a week or more. The method may be used as a treatment to restore cardiac function after a myocardial infarction. The method may also be used to improve blood flow in patients with coronary artery disease suffering from myocardial ischemia or inadequate blood flow to areas other than the heart, for example, peripheral vascular disease, for example, peripheral arterial occlusive disease, where decreased blood flow is a problem.

The compounds can be administered via any medically acceptable means which is suitable for the compound to be administered, including oral, rectal, topical or parenteral (including subcutaneous, intramuscular and intravenous) administration. For example, adenosine has a very short half-life. For this reason, it is preferably administered intravenously. However, adenosine A.sub.2 agonists have been developed which have much longer half-lives, and which can be administered through other means. Thyroid hormone analogs, polymeric forms, and derivatives can be administered, for example, intravenously, oral, topical, intranasal administration.

In some embodiments, the thyroid hormone analogs, polymeric forms, and derivatives are administered via different means.

The amounts of the thyroid hormone, its analogs, polymeric forms, and derivatives required to be effective in stimulating angiogenesis will, of course, vary with the individual being treated and is ultimately at the discretion of the physician. The factors to be considered include the condition of the patient being treated, the efficacy of the particular adenosine A_2 receptor agonist being used, the nature of the formulation, and the patient's body weight. Occlusion-treating dosages of thyroid hormone analogs or its polymeric forms, and derivatives are any dosages that provide the desired effect.

The compounds described above are preferably administered in a formulation including thyroid hormone analogs or its polymeric forms, and derivatives together with an acceptable carrier for the mode of administration. Any formulation or drug delivery system containing the active ingredients, which is suitable for the intended use, as are generally known to those of skill in the art, can be used. Suitable pharmaceutically acceptable carriers for oral, rectal, topical or parenteral (including subcutaneous, intraperitoneal, intramuscular and intravenous) administration are known to those of skill in the art. The carrier must be pharmaceutically acceptable in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

Formulations suitable for parenteral administration conveniently include sterileaqueous preparation of the active compound, which is preferably isotonic with the blood of the recipient. Thus, such formulations may conveniently contain distilled water, 5% dextrose in distilled water or saline. Useful formulations also include concentrated solutions or solids containing the compound of formula (I), which upon dilution with an appropriate solvent give a solution suitable for parental administration above.

For enteral administration, a compound can be incorporated into an inert carrier in discrete units such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of the active compound; as a powder or granules; or a suspension or solution in an aqueous liquid or non-aqueous liquid, e.g., a syrup, an elixir, an emulsion or a draught. Suitable carriers may be starches or sugars and include lubricants, flavorings, binders, and other materials of the same nature.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active compound in a free-flowing form, e.g., a powder or granules, optionally mixed with accessory ingredients, e.g., binders, lubricants, inert diluents, surface active or dispersing agents. Molded tablets may be made by molding in a suitable machine, a mixture of the powdered active compound with any suitable carrier.

A syrup or suspension may be made by adding the active compound to a concentrated, aqueous solution of a sugar, e.g., sucrose, to which may also be added any accessory ingredients. Such accessory ingredients may include flavoring, an agent to retard crystallization of the sugar or an agent to increase the solubility of any other ingredient, e.g., as a polyhydric alcohol, for example, glycerol or sorbitol.

Formulations for rectal administration may be presented as a suppository with a conventional carrier, e.g., cocoa buffer or Witepsol S55 (trademark of Dynamite Nobel Chemical, Germany), for a suppository base.

Alternatively, the compound may be administered in liposomes or microspheres (or microparticles). Methods for preparing liposomes and microspheres for administration to a patient are well known to those of skill in the art. U.S. Pat. No. 4,789,734, the contents of which are hereby incorporated by reference, describes methods for encapsulating biological materials in liposomes. Essentially, the material is dissolved in an aqueous solution, the appropriate phospholipids and lipids added, along with surfactants if required, and the material dialyzed or sonicated, as necessary. A review of known methods is provided by G. Gregoriadis, Chapter 14, "Liposomes," Drug Carriers in Biology and Medicine, pp. 287-341 (Academic Press, 1979).

Microspheres formed of polymers or proteins are well known to those skilled in the art, and can be tailored for passage through the gastrointestinal tract directly into the blood stream. Alternatively, the compound can be incorporated and the microspheres, or composite of microspheres, implanted for slow release over a period of time ranging from days to months. See, for example, U.S. Pat. Nos. 4,906,474, 4,925,673and 3,625,214, and Jein, TIPS 19:155-157(1998), the contents of which are hereby incorporated by reference.

In one embodiment, the thyroid hormone analogs or its polymeric forms, and adenosine derivatives can be formulated into a liposome or microparticle, which is suitably sized to lodge in capillary beds following intravenous administration. When the liposome or microparticle is lodged in the capillary beds surrounding ischemic tissue, the agents can be administered locally to the site at which they can be most effective. Suitable liposomes for targeting ischemic tissue are generally less than about 200nanometers and are also typically unilamellar vesicles, as disclosed, for example, in U.S. Pat. No. 5,593,688 to Baldeschweiler, entitled "Liposomal targeting of ischemic tissue," the contents of which are hereby incorporated by reference.

Preferred microparticles are those prepared from biodegradable polymers, such as polyglycolide, polylactide and copolymers thereof. Those of skill in the art can readily determine an appropriate carrier system depending on various factors, including the desired rate of drug release and the desired dosage.

In one embodiment, the formulations are administered via catheter directly to the inside of blood vessels. The administration can occur, for example, through holes in the catheter.

In those embodiments wherein the active compounds have a relatively long half life (on the order of 1 day to a week or more), the formulations can be included in biodegradable polymeric hydrogels, such as those disclosed in U.S. Pat. No. 5,410,016 to Hubbell et al. These polymeric hydrogels can be delivered to the inside of a tissue lumen and the active compounds released over time as the polymer degrades. If desirable, the polymeric hydrogels can have microparticles or liposomes which include the active compound dispersed therein, providing another mechanism for the controlled release of the active compounds.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the active compound into association with a carrier, which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier or a finely divided solid carrier and then, if necessary, shaping the product into desired unit dosage form.

The formulations can optionally include additional components, such as various biologically active substances such as growth factors (including TGF-.beta., basic fibroblast growth factor (FGF2), epithelial growth factor (EGF), transforming growth factors .alpha. and .beta. (TGF alpha. and beta.), nerve growth factor (NGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor/vascular permeability factor (VEGF/VPF)), antiviral, antibacterial, anti-inflammatory, immuno-suppressant, analgesic, vascularizing agent, and cell adhesion molecule.

In addition to the aforementioned ingredients, the formulations may further include one or more optional accessory ingredient(s) utilized in the art of pharmaceutical formulations, e.g., diluents, buffers, flavoring agents, binders, surface active agents, thickeners, lubricants, suspending agents, preservatives (including antioxidants) and the like.

Formulations and Methods of Treatment

Polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors inducers, or agonists of polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors receptors of the present invention may be administered by any route which is compatible with the particular polymeric thyroid hormone analog alone or in combination with nerve growth factors or other neurogenesis factors, inducer, or agonist employed. Thus, as appropriate, administration may be oral or parenteral, including intravenous and intraperitoneal routes of administration. In addition, administration may be by periodic injections of a bolus of the polymeric thyroid hormone analog alone or in combination with nerve growth factors or other neurogenesis factors, inducer or agonist, or may be made more continuous by intravenous or intraperitoneal administration from a reservoir which is external (e.g., an i.v. bag) or internal (e.g., a bioerodable implant, or a colony of implanted, polymeric thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors-producing cells).

Therapeutic agents of the invention (i.e., polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors, inducers or agonists of polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors receptors) may be provided to an individual by any suitable means, directly (e.g., locally, as by injection, implantation or topical administration to a tissue locus) or systemically (e.g., parenterally or orally). Where the agent is to be provided parenterally, such as by intravenous, subcutaneous, intramolecular, ophthalmic, intraperitoneal, intramuscular, buccal, rectal, vaginal, intraorbital, intracerebral, intracranial, intraspinal, intraventricular, intrathecal, intracisternal, intracapsular, intranasal or by aerosol administration, the agent preferably comprises part of an aqueous or physiologically compatible fluid suspension or solution. Thus, the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors carrier or vehicle is physiologically acceptable so that in addition to delivery of the desired agent to the patient, it does not otherwise adversely affect the patient's electrolyte and/or volume balance. The fluid medium for the agent thus can comprise normal physiologic saline (e.g., 9.85% aqueous NaCl, 0.15M, pH 7-7.4).

Association of the dimer with a polymeric thyroid hormone analog pro domain results in the pro form of the polymeric thyroid hormone analog which typically is more soluble in physiological solutions than the corresponding mature form.

Useful solutions for parenteral administration may be prepared by any of the methods well known in the pharmaceutical art, described, for example, in REMINGTON'S PHARMACEUTICAL SCIENCES (Gennaro, A., ed.), Mack Pub., 1990. Formulations of the therapeutic agents of the invention may include, for example, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. Formulations for direct administration, in particular, may include glycerol and other compositions of high viscosity to help maintain the agent at the desired locus. Biocompatible, preferably bioresorbable, polymers, including, for example, hyaluronic acid, collagen, tricalcium phosphate, polybutyrate, lactide, and glycolide polymers and lactide/glycolide copolymers, may be useful excipients to control the release of the agent in vivo. Other potentially useful parenteral delivery systems for these agents include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation administration contain as excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or oily solutions for administration in the form of nasal drops, or as a gel to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration, methoxysalicylate for rectal administration, or cutric acid for vaginal administration. Suppositories for rectal administration may also be prepared by mixing the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors, inducer or agonist with a non-irritating excipient such as cocoa butter or other compositions which are solid at room temperature and liquid at body temperatures.

Formulations for topical administration to the skin surface may be prepared by dispersing the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors, inducer or agonist with a dermatologically acceptable carrier such as a lotion, cream, ointment or soap. Particularly useful are carriers capable of forming a film or layer over the skin to localize application and inhibit removal. For topical administration to internal tissue surfaces, the agent may be dispersed in a liquid tissue adhesive or other substance known to enhance adsorption to a tissue surface. For example, hydroxypropylcellulose or fibrinogen/thrombin solutions may be used to advantage. Alternatively, tissue-coating solutions, such as pectin-containing formulations may be used.

Alternatively, the agents described herein may be administered orally. Oral administration of proteins as therapeutics

generally is not practiced, as most proteins are readily degraded by digestive enzymes and acids in the mammalian digestive system before they can be absorbed into the bloodstream. However, the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors described herein typically are acid stable and protease-resistant (see, for example, U.S. Pat. No. 4,968,590). In addition, OP-1, has been identified in mammary gland extract, colostrum and 57-day milk. Moreover, the OP-1 purified from mammary gland extract is morphogenically-active and is also detected in the bloodstream. Maternal administration, via ingested milk, may be a natural delivery route of TGF-β superfamily proteins. Letterio, et al., Science 264: 1936-1938 (1994), report that TGF-β is present in murine milk, and that radiolabelled TGF-β is absorbed by gastrointestinal mucosa of suckling juveniles. Labeled, ingested TGF-β appears rapidly in intact form in the juveniles' body tissues, including lung, heart and liver. Finally, soluble form polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors, e.g., mature polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors with or without anti-oxidant or anti-inflammatory agents. These findings, as well as those disclosed in the examples below, indicate that oral and parenteral administration are viable means for administering TGF-β superfamily proteins, including the polymeric thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors, to an individual. In addition, while the mature forms of certain polymeric thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors described herein typically are sparingly soluble, the polymeric thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors form found in milk (and mammary gland extract and colostrum) is readily soluble, probably by association of the mature, morphogenically-active form with part or all of the pro domain of the expressed, full length polypeptide sequence and/or by association with one or more milk components. Accordingly, the compounds provided herein may also be associated with molecules capable of enhancing their solubility in vitro or in vivo.

Where the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors is intended for use as a therapeutic for disorders of the CNS, an additional problem must be addressed: overcoming the blood-brain barrier, the brain capillary wall structure that effectively screens out all but selected categories of substances present in the blood, preventing their passage into the brain. The blood-brain barrier can be bypassed effectively by direct infusion of the polymeric thyroid hormone analogs into the brain, or by intranasal administration or inhalation of formulations suitable for uptake and retrograde transport by olfactory neurons. Alternatively, the polymeric thyroid hormone analogs can be modified to enhance its transport across the blood-brain barrier. For example, truncated forms of the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors or a polymeric thyroid hormone analog alone or in combination with nerve growth factor or other neurogenesis factors-stimulating agent may be most successful. Alternatively, the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors, inducers or agonists provided herein can be derivatized or conjugated to a lipophilic moiety or to a substance that is actively transported across the blood-brain barrier, using standard means known to those skilled in the art. See, for example, Pardridge, Endocrine Reviews 7: 314-330 (1986) and U.S. Pat. No. 4,801,575.

The compounds provided herein may also be associated with molecules capable of targeting the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors, inducer or agonist to the desired tissue. For example, an antibody, antibody fragment, or other binding protein that interacts specifically with a surface molecule on cells of the desired tissue, may be used. Useful targeting molecules may be designed, for example, using the single chain binding site technology disclosed in U.S. Pat. No. 5,091,513. Targeting molecules can be covalently or non-covalently associated with the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors, inducer or agonist.

As will be appreciated by one of ordinary skill in the art, the formulated compositions contain therapeutically-effective amounts of the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors, inducers or agonists thereof. That is, they contain an amount which provides appropriate concentrations of the agent to the affected nervous system tissue for a time sufficient to stimulate a detectable restoration of impaired central or peripheral nervous system function, up to and including a complete restoration thereof. As will be appreciated by those skilled in the art, these concentrations will vary depending upon a number of factors, including the biological efficacy of the selected agent, the chemical characteristics (e.g., hydrophobicity) of the specific agent, the formulation thereof, including a mixture with one or more excipients, the administration route, and the treatment envisioned, including whether the active ingredient will be administered directly into a tissue site, or whether it will be administered systemically. The preferred dosage to be administered is also likely to depend on variables such as the condition of the diseased or damaged tissues, and the overall health status of the particular mammal. As a general matter, single, daily, biweekly or weekly dosages of 0.00001-1000 mg of a polymeric thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors are sufficient in the presence of anti-oxidant and/or anti-inflammatory agents, with 0.0001-100 mg being preferable, and 0.001 to 10 mg being even more preferable. Alternatively, a single, daily, biweekly or weekly dosage of 0.01-1000 .mu.g/kg body weight, more preferably 0.01-10 mg/kg body weight, may be advantageously employed. The present effective dose can be administered in a single dose or in a plurality (two or more) of installment doses, as desired or considered appropriate under the specific circumstances. A bolus injection or diffusable infusion formulation can be used. If desired to facilitate repeated or frequent infusions, implantation of a semi-permanent stent (e.g., intravenous, intraperitoneal, intracisternal or intracapsular) may be advisable.

The polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors, inducers or agonists of the invention may, of course, be administered alone or in combination with other molecules known to be beneficial in the treatment of the conditions described herein. For example, various well-known growth factors, hormones, enzymes, therapeutic compositions, antibiotics, or other bioactive agents can also be administered with the polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors. Thus, various known growth factors such as NGF, EGF, PDGF, IGF, FGF, TGF-α, and TGF-β, as well as

enzymes, enzyme inhibitors, antioxidants, anti-inflammatory agents, free radical scavenging agents, antibiotics and/or chemoattractant/chemotactic factors, can be included in the present polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors formulation.

Materials & Methods

Reagents: All reagents were chemical grade and purchased from Sigma Chemical Co. (St. Louis, Mo.) or through VWR Scientific (Bridgeport, N.J.). Cortisone acetate, bovine serum albumin (BSA) and gelatin solution (2% type B from bovine skin) were purchased from Sigma Chemical Co. Fertilized chicken eggs were purchased from Charles River Laboratories, SPAFAS Avian Products & Services (North Franklin, Conn.). T4, 3,5,3'-triiodo-L-thyronine (T3), tetraiodothyroacetic acid (tetrac), T4-agarose, 6-N-propyl-2-thiouracil (PTU), RGD-containing peptides, and RGE-containing peptides were obtained from Sigma; PD 98059 from Calbiochem; and CGP41251 was a gift from Novartis Pharma (Basel, Switzerland). Polyclonal anti-FGF2 and monoclonal anti-β-actin were obtained from Santa Cruz Biotechnology and human recombinant FGF2 and VEGF from Invitrogen. Polyclonal antibody to phosphorylated ERK1/2 was from New England Biolabs and goat anti-rabbit IgG from DAKO. Monoclonal antibodies to αVβ3 (SC73 12) and α-tubulin (E9) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Normal mouse IgG and HRP-conjugated goat anti-rabbit Ig were purchased from Dako Cytomation (Carpinteria, Calif.). Monoclonal antibodies to αVβ3 (LM609) and αVβ5 (P1F6), as well as purified αVβ3, were purchased from Chemicon (Temecula, Calif.). L-[^{125}I]-T4 (specific activity, 1250 μCi/μg) was obtained from Perkin Elmer Life Sciences (Boston, Mass.).

Chorioallantoic membrane (CAM) Model of Angiogenesis: In vivo Neovascularization was examined by methods described previously. 9-12 Ten-day-old chick embryos were purchased from SPAFAS (Preston, Conn.) and incubated at 37° C. with 55% relative humidity. A hypodermic needle was used to make a small hole in the shell concealing the air sac, and a second hole was made on the broad side of the egg, directly over an avascular portion of the embryonic membrane that was identified by candling. A false air sac was created beneath the second hole by the application of negative pressure at the first hole, causing the CAM to separate from the shell. A window approximately 1.0 cm 2 was cut in the shell over the dropped CAM with a small-crafts grinding wheel (Dremel, division of Emerson Electric Co.), allowing direct access to the underlying CAM. FGF2 (1 μg/mL) was used as a standard proangiogenic agent to induce new blood vessel branches on the CAM of 10-day-old embryos. Sterile disks of No. 1 filter paper (Whatman International) were pretreated with 3 mg/mL cortisone acetate and 1 mmol/L PTU and air dried under sterile conditions. Thyroid hormone, hormone analogues, FGF2 or control solvents, and inhibitors were then applied to the disks and the disks allowed to dry. The disks were then suspended in PBS and placed on growing CAMs. Filters treated with T4 or FGF2 were placed on the first day of the 3-day incubation, with antibody to FGF2 added 30 minutes later to selected samples as indicated. At 24 hours, the MAPK cascade inhibitor PD 98059 was also added to CAMs topically by means of the filter disks.

Microscopic Analysis of CAM Sections: After incubation at 37° C. with 55% relative humidity for 3 days, the CAM tissue directly beneath each filter disk was resected from control and treated CAM samples. Tissues were washed 3× with PBS, placed in 35-mm Petri dishes (Nalge Nunc), and examined under an SV6 stereomicroscope (Zeiss) at ×50 magnification. Digital images of CAM sections exposed to filters were collected using a 3-charge-coupled device color video camera system (Toshiba) and analyzed with Image-Pro software (Media Cybernetics). The number of vessel branch points contained in a circular region equal to the area of each filter disk were counted. One image was counted in each CAM preparation, and findings from 8 to 10 CAM preparations were analyzed for each treatment condition (thyroid hormone or analogues, FGF2, FGF2 antibody, PD 98059). In addition, each experiment was performed 3 times. The resulting angiogenesis index is the mean ±SEM of new branch points in each set of samples.

FGF2 Assays: ECV304 endothelial cells were cultured in M199 medium supplemented with 10% fetal bovine serum. ECV304 cells (10^6 cells) were plated on 0.2% gel-coated 24-well plates in complete medium overnight, and the cells were then washed with serum-free medium and treated with T4 or T3 as indicated. After 72 hours, the supernatants were harvested and assays for FGF performed without dilution using a commercial ELISA system (R&D Systems).

MAPK Activation: ECV304 endothelial cells were cultured in M199 medium with 0.25% hormone-depleted serum 13 for 2 days. Cells were then treated with T4 (10^{-7} mol/L) for 15 minutes to 6 hours. In additional experiments, cells were treated with T4 or FGF2 or with T4 in the presence of PD 98059 or CGP41251. Nuclear fractions were pre-pared from all samples by our method reported previously, the proteins separated by polyacrylamide gel electrophoresis, and transferred to membranes for immunoblotting with antibody to phosphorylated ERK 1/2. The appearance of nuclear phosphorylated ERK1/2 signifies activation of these MAPK isoforms by T4.

Reverse Transcription-Polymerase Chain Reaction: Confluent ECV304 cells in 10-cm plates were treated with T4 (10^{-7} mol/L) for 6 to 48 hours and total RNA extracted using guanidinium isothiocyanate (Biotecx Laboratories). RNA (1 μg) was subjected to reverse transcription-polymerase chain reaction (RT-PCR) using the Access RT-PCR system (Promega). Total RNA was reverse transcribed into cDNA at 48° C. for 45 minutes, then denatured at 94° C. for 2 minutes. Second-strand synthesis and PCR amplification were performed for 40 cycles with denaturation at 94° C. for 30 s, annealing at 60° C. for 60 s, and extension at 68° C. for 120 s, with final ex-tension for 7 minutes at 68° C. after completion of all cycles. PCR primers for FGF2 were as follows: FGF2 sense strand 5'-TGGTATGTGGCACTGAAACG-3' (SEQ ID NO:1), antisense strand 5' CTCAATGACCTGGCGAAGAC-3' (SEQ ID NO:2); the length of the PCR product was 734 bp. Primers for GAPDH included the sense strand 5'-AAGGTCATCCCTGAGCTGAACG-3' (SEQ ID NO:3), and antisense strand 5'-GGGTGTCGCTGTTGAAGTCAGA-3' (SEQ ID NO:4); the length of the PCR product was 218 bp. The products of RT-PCR were separated by electrophoresis on 1.5% agarose gels and visualized with ethidium bromide. The target bands of the gel were quantified using LabImage software (Kapelan), and the value for [FGF2/GAPDH]X10 calculated for each time point.

Statistical Analysis: Statistical analysis was performed by 1-way analysis of variance (ANOVA) comparing experimental with respective control group and statistical significance was calculated based on $P<0.05$.

In vivo angiogenesis in Matrigel FGF_2 or Cancer cell lines implant in mice: In Vivo Murine Angiogenesis Model: The murine matrigel model will be conducted according to previously described methods (Grant et al., 1991; Okada et al., 1995) and as implemented in our laboratory (Powel et al., 2000). Briefly, growth factor free matrigel (Becton Dickin-

son, Bedford Mass.) will be thawed overnight at 4° C. and placed on ice. Aliquots of matrigel will be placed into cold polypropylene tubes and FGF2, thyroid hormone analogs or cancer cells (1×10^6 cells) will be added to the matrigel. Matrigel with Saline, FGF2, thyroid hormone analogs or cancer cells will be subcutaneously injected into the ventral midline of the mice. At day 14, the mice will be sacrificed and the solidified gels will be resected and analyzed for presence of new vessels. Compounds A-D will be injected subcutaneously at different doses. Control and experimental gel implants will be placed in a micro centrifuge tube containing 0.5 ml of cell lysis solution (Sigma, St. Louis, Mo.) and crushed with a pestle. Subsequently, the tubes will be allowed to incubate overnight at 4° C. and centrifuged at 1,500×g for 15 minutes on the following day. A 200 μl aliquot of cell lysate will be added to 1.3 ml of Drabkin's reagent solution (Sigma, St. Louis, Mo.) for each sample. The solution will be analyzed on a spectrophotometer at a 540 nm. The absorption of light is proportional to the amount of hemoglobin contained in the sample.

Tumor growth and metastasis—Chick Chorioallantoic Membrane (CAM) model of tumor implant: The protocol is as previously described (Kim et al., 2001). Briefly, 1×10^7 tumor cells will be placed on the surface of each CAM (7 day old embryo) and incubated for one week. The resulting tumors will be excised and cut into 50 mg fragments. These fragments will be placed on additional 10 CAMs per group and treated topically the following day with 25 μl of compounds (A-D) dissolved in PBS. Seven days later, tumors will then be excised from the egg and tumor weights will be determined for each CAM. FIG. **8** is a diagrammatic sketch showing the steps involved in the in vivo tumor growth model in the CAM.

The effects of TETRAC, TRIAC, and thyroid hormone antagonists on tumor growth rate, tumor angiogenesis, and tumor metastasis of cancer cell lines can be determined.

Tumor growth and metastasis—Tumor Xenograft model in mice: The model is as described in our publications by Kerr et al., 2000; Van Waes et al., 2000; Ali et al., 2001; and Ali et al., 2001, each of which is incorporated herein by reference in its entirety). The anti-cancer efficacy for TETRAC, TRIAC, and other thyroid hormone antagonists at different doses and against different tumor types can be determined and compared.

Tumor growth and metastasis—Experimental Model of Metastasis: The model is as described in our recent publications (Mousa, 2002; Amirkhosravi et al., 2003a and 2003b, each of which is incorporated by reference herein in its entirety). Briefly, B16 murine malignant melanoma cells (ATCC, Rockville, Md.) and other cancer lines will be cultured in RPMI 1640 (Invitrogen, Carlsbad, Calif.), supplemented with 10% fetal bovine serum, penicillin and streptomycin (Sigma, St. Louis, Mo.). Cells will be cultured to 70% confluency and harvested with trypsin-EDTA (Sigma) and washed twice with phosphate buffered saline (PBS). Cells will be re-suspended in PBS at a concentration of either 2.0×10^5 cells/ml for experimental metastasis. Animals: C57/BL6 mice (Harlan, Indianapolis, Ind.) weighing 18-21 grams will be used for this study. All procedures are in accordance with IACUC and institutional guidelines. The anti-cancer efficacy for TETRAC, TRIAC, and other thyroid hormone antagonists at different doses and against different tumor types can be determined and compared.

Effect of Thyroid Hormone Analogues on Angiogenesis

T4 induced significant increase in angiogenesis index (fold increase above basal) in the CAM model. T3 at 0.001-1.0 μM or T4 at 0.1-1.0 μM achieved maximal effect in producing 2-2.5 fold increase in angiogenesis index as compared to 2-3 fold increase in angiogenesis index by 1 μg of FGF2 (Table 1 and FIG. **1***a* and **1***b*). The effect of T4 in promoting angiogenesis (2-2.5 fold increase in angiogenesis index) was achieved in the presence or absence of PTU, which inhibit T4 to T3 conversion. T3 itself at 91-100 nM)-induced potent pro-angiogenic effect in the CAM model. T4 agarose produced similar pro-angiogenesis effect to that achieved by T4. The pro-angiogenic effect of either T4 or T4-agarose was 100% blocked by TETRAC or TRIAC.

Enhancement of Pro-Angiogenic Activity of FGF2 By Sub-Maximal Concentrations of T_4.

The combination of T4 and FGF2 at sub-maximal concentrations resulted in an additive increase in the angiogenesis index up to the same level like the maximal pro-angiogenesis effect of either FGF2 or T4 (FIG. **2**).

Effects of MAPK cascade inhibitors on the pro-angiogenic actions of T_4 and FGf2 n the CAM model. The pro-angiogenesis effect of either T4 or FGF2 was totally blocked by PD 98059 at 0.8-8 μg (FIG. **3**).

Effects of specific integrin αVβ3 antagonists on the pro-angiogenic actions of T_4 and FGf2 n the CAM model. The pro-angiogenesis effect of either T4 or FGF2 was totally blocked by the specific monoclonal antibody LM609 at 10 μg (FIGS. **4***a* and **4***b*).

The CAM assay has been used to validate angiogenic activity of a variety of growth factors and other promoters or inhibitors of angiogenesis. In the present studies, T_4 in physiological concentrations was shown to be pro-angiogenic, with comparable activity to that of FGF2. The presence of PTU did not reduce the effect of T_4, indicating that de-iodination of T_4 to generate T_3 was not a prerequisite in this model. Because the appearance of new blood vessel growth in this model requires several days, we assumed that the effect of thyroid hormone was totally dependent upon the interaction of the nuclear receptor for thyroid hormone (TR). Actions of iodothyronines that require intranuclear complexing of TR with its natural ligand, T_3, are by definition, genomic, and culminate in gene expression. On the other hand, the preferential response of this model system to T_4-rather than T_3, the natural ligand of TR raised the possibility that angiogenesis might be initiated non-gnomically at the plasma membrane by T_4 and culminate in effects that require gene transcription. Non-genomic actions of T_4 have been widely described, are usually initiated at the plasma membrane and may be mediated by signal transduction pathways. They do not require intranuclear ligand binding of iodothyronine and TR, but may interface with or modulate gene transcription. Non-genomic actions of steroids have also been well-described and are known to interface with genomic actions of steroids or of other compounds. Experiments carried out with T_4 and tetrac or with agarose-T_4 indicated that the pro-angiogenic effect of T_4 indeed very likely was initiated at the plasma membrane. We have shown elsewhere that tetrac blocks membrane-initiated effects of T_4, but does not, itself, activate signal transduction. Thus, it is a probe for non-genomic actions of thyroid hormone. Agarose-T_4 is thought not to gain entry to the cell interior and has been used by us and others to examine models for possible cell surface-initiated actions of the hormone.

These results suggest that another consequence of activation of MAPK by thyroid hormone is new blood vessel growth. The latter is initiated nongenomically, but of course requires a consequent complex gene transcription program.

The ambient concentrations of thyroid hormone are relatively stable. The CAM model, at the time we tested it, was thyroprival and thus may be regarded as a system, which does not reproduce the intact organism. We propose that circulat-

ing levels of T_4 serve, with a variety of other regulators, to modulate the sensitivity of vessels to endogenous angiogenic factors, such as VEGF and FGF2.

Three-Dimensional Angiogenesis Assay

In Vitro Three-Dimensional Sprout Angiogenesis of Human Dermal Micro-Vascular Endothelial Cells (HDMEC) Cultured on Micro-Carrier Beads Coated with Fibrin: Confluent HDMEC (passages 5-10) were mixed with gelatin-coated Cytodex-3 beads with a ratio of 40 cells per bead. Cells and beads (150-200 beads per well for 24-well plate) were suspended with 5 ml EBM+15% normal human serum, mixed gently every hour for first 4 hours, then left to culture in a CO_2 incubator overnight. The next day, 10 ml of fresh EBM+5% HS were added, and the mixture was cultured for another 3 hours. Before experiments, the culture of EC-beads was checked; then 500 ul of PBS was added to a well of 24-well plate, and 100 ul of the EC-bead culture solution was added to the PBS. The number of beads was counted, and the concentration of EC/beads was calculated.

A fibrinogen solution (1 mg/ml) in EBM medium with or without angiogenesis factors or testing factors was prepared. For positive control, 50 ng/ml VEGF+25 ng/ml FGF2 was used. EC-beads were washed with EBM medium twice, and EC-beads were added to fibrinogen solution. The experiment was done in triplicate for each condition. The EC-beads were mixed gently in fibrinogen solution, and 2.5 ul human thrombin (0.05 U/ul) was added in 1 ml fibrinogen solution; 300 ul was immediately transferred to each well of a 24-well plate. The fibrinogen solution polymerizes in 5-10 minutes; after 20 minutes, we added EBM+20% normal human serum+10 ug/ml aprotinin. The plate was incubated in a CO_2 incubator. It takes about 24-48 hours for HDMEC to invade fibrin gel and form tubes.

A micro-carrier in vitro angiogenesis assay previously designed to investigate bovine pulmonary artery endothelial cell angiogenic behavior in bovine fibrin gels [Nehls and Drenckhaln, 1995a, b] was modified for the study of human microvascular endothelial cell angiogenesis in three-dimensional ECM environments (FIGS. **1** and **2**). Briefly, human fibrinogen, isolated as previously described [Feng et al, 1999], was dissolved in M199 medium at a concentration of 1 mg/ml (pH 7.4) and sterilized by filtering through a 0.22 micron filter. An isotonic 1.5 mg/ml collagen solution was prepared by mixing sterile Vitrogen 100 in 5×M199 medium and distilled water. The pH was adjusted to 7.4 by 1N NaOH. In certain experiments, growth factors and ECM proteins (such as VEGF, bFGF, PDGF-BB, serum, gelatin, and fibronectin) were added to the fibrinogen or collagen solutions. About 500 EC-beads were then added to the 1 mg/ml fibrinogen or 1.5 mg/ml collagen solutions. Subsequently, EC-beads-collagen or EC-beads-fibrinogen suspension (500 EC-beads/ml) was plated onto 24-well plates at 300 ul/well. EC-bead-collagen cultures were incubated at 37° C. to form gel. The gelling of EC-bead-fibrin cultures occurred in less than 5 minutes at room temperature after the addition of thrombin to a final concentration of 0.5 U/ml. After gelation, 1 ml of fresh assay medium (EBM supplemented with 20% normal human serum for HDMEC or EBM supplemented with 10% fetal bovine serum was added to each well. The angiogenic response was monitored visually and recorded by video image capture. Specifically, capillary sprout formation was observed and recorded with a Nikon Diaphot-TMD inverted microscope (Nikon Inc.; Melville, N.Y.), equipped with an incubator housing with a Nikon NP-2 thermostat and Sheldon #2004 carbon dioxide flow mixer. The microscope was directly interfaced to a video system consisting of a Dage-MTI CCD-72S video camera and Sony 12" PVM-122 video monitor linked to a Macintosh G3 computer. The images were captured at various magnifications using Adobe Photoshop. The effect of angiogenic factors on sprout angiogenesis was quantified visually by determining the number and percent of EC-beads with capillary sprouts. One hundred beads (five to six random low power fields) in each of triplicate wells were counted for each experimental condition. All experiments were repeated at least three times.

Cell Culture

The African green monkey fibroblast cell line, CV-1 (ATCC, Manassas, Va.), which lacks the nuclear receptor for thyroid hormone, was plated at 5000 cells/cm^2 and maintained in DMEM, supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. All culture reagents were purchased from Invitrogen Corporation (Carlsbad, Calif.). Cultures were maintained in a 37° C. humidified chamber with 5% CO_2. The medium was changed every three days and the cell lines were passaged at 80% confluency. For experimental treatment, cells were plated in 10-cm cell culture dishes (Corning Incorporated, Corning, N.Y.) and allowed to grow for 24 h in 10% FBS-containing medium. The cells were then rinsed twice with phosphate buffered saline (PBS) and fed with serum-free DMEM supplemented with penicillin, streptomycin, and HEPES. After 48 h incubation in serum-free media, the cells were treated with a vehicle control (final concentration of 0.004 N KOH with 0.4% polyethyleneglycol [v/v]) or T4 (10^{-7} M final concentration) for 30 min; media were then collected and free T4 levels were determined by enzyme immunoassays. Cultures incubated with 10^{-7} M total T4 have 10^{-9} to 10^{-10} M free T4. Following treatment, the cells were harvested and the nuclear proteins prepared as previously described.

Transient Transfections with siRNA

CV-1 cells were plated in 10-cm dishes (150,000 cells/dish) and incubated for 24 h in DMEM supplemented with 10% FBS. The cells were rinsed in OPTI-MEM (Ambion, Austin, Tex.) and transfected with siRNA (100 nM final concentration) to αV, β3, or αV and β3 together using siPORT (Ambion) according to manufacturer's directions. Additional sets of CV-1 cells were transfected with a scrambled siRNA, to serve as a negative control. Four hours post-transfection, 7 ml of 10% FBS-containing media was added to the dishes and the cultures were allowed to incubate overnight. The cells were then rinsed with PBS and placed in serum-free DMEM for 48 h before treatment with T4.

RNA Isolation and RT-PCR

Total RNA was extracted from cell cultures 72 h post-transfection using the RNeasy kit from Qiagen (Valencia, Calif.) as per manufacturer's instructions. Two hundred nanograms of total RNA was reverse-transcribed using the Access RT-PCR system (Promega, Madison, Wis.) according to manufacturer's directions. Primers were based on published species-specific sequences: αV (accession number NM-002210) F-5'-TGGGATTGTGGAAGGAG and R-5'-AAATCCCTGTCCATCAGCAT (319 bp product), β3 (NM000212) F-5'-GTGTGAGTGCTCAGAGGAG and R-5'-CTGACTCAATCTCGTCACGG (5 15 bp product), and GAPDH (AF261085) F-5'-GTCAGTGGTGGACCTGACCT and R-5'-TGAGCTTGACMGTGGTCG (212 bp product). RT-PCR was performed in the Flexigene thermal cycler eom TECHNE (Burlington, N.J.). After a 2 min incubation at 95° C., 25 cycles of the following steps were performed: denaturation at 94° C. for 1 min, annealing at 57° C. for 1 min, and extension for 1 min at 68° C. for 25 cycles. The PCR products were visualized on a 1.8% (w/v) agarose gel stained with ethidium bromide.

Western Blotting

Aliquots of nuclear proteins (10 μg/lane) were mixed with Laemmli sample buffer and separated by SDS-PAGE (10% resolving gel) and then transferred to nitrocellulose membranes. After blocking with 5% non-fat milk in Tris-buffered saline containing 1% Tween-20 (TBST) for 30 min, the membranes were incubated with a 1:1000 dilution of a monoclonal antibody to phosphorylated p44/42 MAP kinase (Cell Signaling Technology, Beverly, Mass.) in TBST with 5% milk overnight at 4° C. Following 3×10-min washes in TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit Ig (1:1000 dilution) from DakoCytomation (Carpinteria, Calif.) in TBST with 5% milk for 1 h at room temperature. The membranes were washed 3×5 min in TBST and immunoreactive proteins were detected by chemiluminescence (ECL, Amersham). Band intensity was determined using the VersaDoc 5000 Imaging system (Bio-Rad, Hercules, Calif.).

Radioligand Binding Assay

Two μg of purified αVβ3 was mixed with indicated concentrations of test compounds and allowed to incubate for 30 min at room temperature. [^{125}I]-T4 (2 μCi) was then added and the mixture was allowed to incubate an additional 30 min at 20° C. The samples were mixed with sample buffer (50% glycerol, 0.1M Tris-HCl, pH 6.8, and bromophenol blue) and runout on a 5% basic-native gel for 24 h at 45 mA in the cold. The apparatus was disassembled and the gels were placed on filter paper, wrapped in plastic wrap, and exposed to film. Band intensity was determined using the VersaDoc 5000 Imaging system.

Chick Chorioallantoic Membrane (CAM) Assay (αVβ3 Studies)

Ten-day-old chick embryos were purchased £tom SPAFAS (Preston, Conn.) and were incubated at 37° C. with 55% relative humidity. A hypodermic needle was used to make a small hole in the blunt end of the egg and a second hole was made on the broad side of the egg, directly over an avascular portion of the embryonic membrane. Mild suction was applied to the first hole to displace the air sac and drop the CAM away from the shell. Using a Dremel model craft drill (Dremel, Racine, Wis.), a approx. 1.0 cm^2 window was cut in the shell over the false air sac, allowing access to the CAM. Sterile disks of No. 1 filter paper (Whatman, Clifton, N.J.) were pretreated with 3 mg/ml cortisone acetate and 1mnM-mpropylthiouracil and air dried under sterile conditions. Thyroid hormone, control solvents, and the mAb LM609 were applied to the disks and subsequently dried. The disks were then suspended in PBS and placed on growing CAMS. After incubation for 3 days, the CAM beneath the filter disk was resected and rinsed with PBS. Each membrane was placed in a 35 mm Petri dish and examined under an SV6 stereomicroscope at 50× magnification. Digital images were captured and analyzed with Image-Pro software (Mediacybemetics). The number of vessel branch points contained in a circular region equal to the filter disk were counted. One image from each of 8-10 CAM preparations for each treatment condition was counted, and in addition each experiment was performed 3 times.

The invention will be further illustrated in the following non-limiting examples.

EXAMPLES

Example 1

Effect of Thyroid Hormone on Angiogenesis

As seen in FIG. **1**A and summarized in FIG. **1**B, both L-T4 and L-T3 enhanced angiogenesis in the CAM assay. T4, at a physiologic total concentration in the medium of 0.1 μmol/L, increased blood vessel branch formation by 2.5-fold ($P<0.001$). T3 (1 nmol/L) also stimulated angiogenesis 2-fold. The possibility that T4 was only effective because of conversion of T4 to T3 by cellular 5'-monodeiodinase was ruled out by the finding that the deiodinase inhibitor PTU had no inhibitory effect on angiogenesis produced by T4. PTU was applied to all filter disks used in the CAM model. Thus, T4 and T3 promote new blood vessel branch formation in a CAM model that has been standardized previously for the assay of growth factors.

Example 2

Effects of T4-Agarose and Tetrac

We have shown previously that T4-agarose stimulates cellular signal transduction pathways initiated at the plasma membrane in the same manner as T4 and that the actions of T4 and T4-agarose are blocked by a deaminated iodothyronine analogue, tetrac, which is known to inhibit binding of T4 to plasma membranes. In the CAM model, the addition of tetrac (0.1 μmol/L) inhibited the action of T4 (FIG. **2**A), but tetrac alone had no effect on angiogenesis (FIG. **2**C). The action of T4-agarose, added at a hormone concentration of 0.1 μmol/L, was comparable to that of T4 in the CAM model (FIG. **2**B), and the effect of T4-agarose was also inhibited by the action of tetrac (FIG. **2**B; summarized in **2**C).

Example 3

Enhancement of Proangiogenic Activity of FGF2 by a Submaximal Concentration of T4

Angiogenesis is a complex process that usually requires the participation of polypeptide growth factors. The CAM assay requires at least 48 hours for vessel growth to be manifest; thus, the apparent plasma membrane effects of thyroid hormone in this model are likely to result in a complex transcriptional response to the hormone. Therefore, we determined whether FGF2 was involved in the hormone response and whether the hormone might potentiate the effect of subphysiologic levels of this growth factor. T4 (0.05 μmol/L) and FGF2 (0.5 μg/mL) individually stimulated angiogenesis to a modest degree (FIG. **3**). The angiogenic effect of this submaximal concentration of FGF2 was enhanced by a subphysiologic concentration of T4 to the level caused by 1.0 μg FGF2 alone. Thus, the effects of submaximal hormone and growth factor concentrations appear to be additive. To define more precisely the role of FGF2 in thyroid hormone stimulation of angiogenesis, a polyclonal antibody to FGF2 was added to the filters treated with either FGF2 or T4, and angiogenesis was measured after 72 hours. FIG. **4** demonstrates that the FGF2 antibody inhibited angiogenesis stimulated either by FGF2 or by T4 in the absence of exogenous FGF2, suggesting that the T4 effect in the CAM assay was mediated by increased FGF2 expression. Control IgG antibody has no stimulatory or inhibitory effect in the CAM assay.

Example 4

Stimulation of FGF2 Release From Endothelial Cells by Thyroid Hormone

Levels of FGF2 were measured in the media of ECV304 endothelial cells treated with either T4 (0.1 μmol/L) or T3 (0.01 μmol/L) for 3 days. As seen in the Table 2, T3 stimulated

FGF2 concentration in the medium 3.6-fold, whereas T4 caused a 1.4-fold increase. This finding indicates that thyroid hormone may enhance the angiogenic effect of FGF2, at least in part, by increasing the concentration of growth factor available to endothelial cells.

TABLE 2

Effect of T4 and T3 on Release of FGF2 From ECV304 Endothelial Cells

Cell Treatment	FGF2 (pg/mL/10^6 cells)
Control	27.7 ± 3.1
T3 (0.01 μmol/L)	98.8 ± 0.5*
T3 + PD 98059 (2 μmol/L)	28.4 ± 3.2
T3 + PD 98059 (20 μmol/L)	21.7 ± 3.5
T4 (0.1 μmol/L)	39.2 ± 2.8†
T4 + PD 98059 (2 μmol/L)	26.5 ± 4.5
T4 + PD 98059 (20 μmol/L)	23.2 ± 4.8

*$P < 0.001$, comparing T3-treated samples with control samples by ANOVA;
†$P < 0.05$, comparing T4-treated samples with control samples by ANOVA.

Example 5

Role of the ERK1/2 Signal Transduction Pathway in Stimulation of Angiogenesis by Thyroid Hormone and FGF2

A pathway by which T4 exerts a nongenomic effect on cells is the MAPK signal transduction cascade, specifically that of ERK1/2 activation. We know that T4 enhances ERK1/2 activation by epidermal growth factor. The role of the MAPK pathway in stimulation by thyroid hormone of FGF2 expression was examined by the use of PD 98059 (2 to 20 μmol/L), an inhibitor of ERK1/2 activation by the tyrosine-threonine kinases MAPK kinase-1 (MEK1) and MEK. The data in the Table demonstrate that PD 98059 effectively blocked the increase in FGF2 release from ECV304 endothelial cells treated with either T4 or T3. Parallel studies of ERK1/2 inhibition were performed in CAM assays, and representative results are shown in FIG. **5**. A combination of T3 and T4, each in physiologic concentrations, caused a 2.4-fold increase in blood vessel branching, an effect that was completely blocked by 3 μmol/L PD 98059 (FIG. **5**A). FGF2 stimulation of branch formation (2.2-fold) was also effectively blocked by this inhibitor of ERK1/2 activation (FIG. **5**B). Thus, the proangiogenic effect of thyroid hormone begins at the plasma membrane and involves activation of the ERK1/2 pathway to promote FGF2 release from endothelial cells. ERK1/2 activation is again required to transduce the FGF2 signal and cause new blood vessel formation.

Example 6

Action of Thyroid Hormone and FGF2 on MAPK Activation

Stimulation of phosphorylation and nuclear translocation of ERK1/2 MAPKs was studied in ECV304 cells treated with T4 (10^{-7} mol/L) for 15 minutes to 6 hours. The appearance of phosphorylated ERK1/2 in cell nuclei occurred within 15 minutes of T4 treatment, reached a maximal level at 30 minutes, and was still apparent at 6 hours (FIG. **6**A). This effect of the hormone was inhibited by PD 98059 (FIG. **6**B), a result to be expected because this compound blocks the phosphorylation of ERK1/2 by MAPK kinase. The traditional protein kinase C (PKC)-α, PKC-β, and PKC-γ inhibitor CGP41251 also blocked the effect of the hormone on MAPK activation in these cells, as we have seen with T4 in other cell lines. Thyroid hormone enhances the action of several cytokines and growth factors, such as interferon-γ13 and epidermal growth factor. In ECV304 cells, T4 enhanced the MAPK activation caused by FGF2 in a 15-minute co incubation (FIG. **6**C). Applying observations made in ECV304 cells to the CAM model, we propose that the complex mechanism by which the hormone induces angiogenesis includes endothelial cell release of FGF2 and enhancement of the autocrine effect of released FGF2 on angiogenesis.

Example 7

RT-PCR in ECV304 Cells Treated with Thyroid Hormone

The final question addressed in studies of the mechanism of the proangiogenic action of T4 was whether the hormone may induce FGF2 gene expression. Endothelial cells were treated with T4 (10^{-7} mol/L) for 6 to 48 hours, and RT-PCR-based estimates of FGF2 and GAPDH RNA (inferred from cDNA measurements; FIG. **7**) were performed. Increase in abundance of FGF2 cDNA, corrected for GAPDH content, was apparent by 6 hours of hormone treatment and was further enhanced by 48 hours.

Example 8A

Retinal Neovascularization Model in Mice (Diabetic and Non-Diabetic)

To assess the pharmacologic activity of a test article on retinal neovascularization, Infant mice are exposed to a high oxygen environment for 7 days and allowed to recover, thereby stimulating the formation of new vessels on the retina. Test articles are evaluated to determine if retinal neovascularization is suppressed. The retinas are examined with hematoxylin-eosin staining and with at least one stain, which demonstrates neovascularization (usually a Selectin stain). Other stains (such as PCNA, PAS, GFAP, markers of angiogenesis, etc.) can be used. A summary of the model is below:

Animal Model

Infant mice (P7) and their dams are placed in a hyperoxygenated environment (70-80%) for 7 days.
On P12, the mice are removed from the oxygenated environment and placed into a normal environment
Mice are allowed to recover for 5-7 days.
Mice are then sacrificed and the eyes collected.
Eyes are either frozen or fixed as appropriate
The eyes are stained with appropriate histochemical stains
The eyes are stained with appropriate immunohistochemical stains
Blood, serum, or other tissues can be collected
Eyes, with special reference to microvascular alterations, are examined for any and all findings. Neovascular growth will be semi quantitatively scored. Image analysis is also available.

Example 8B

Thyroid Hormone and Diabetic Retinopathy

A protocol disclosed in J de la Cruz et al., J Pharmacol Exp Ther 280:454-459, 1997, is used for the administration of

Tetrac to rats that have streptozotocin (STZ)-induced experimental diabetes and diabetic retinopathy. The endpoint is the inhibition by Tetrac of the appearance of proliferative retinopathy (angiogenesis).

Example 9A

Wound Healing and Hemostatic Treatment Using Novel Pharmaceutical Polymeric Formulation of Thyroid Hormone and Analogs

The present invention also includes a novel wound healing and hemostatic treatment that include an immobilized thyroid hormone analog, preferably T4 analogs, calcium chloride, and collagen. This novel formulation significantly controls both venous and arterial hemorrhage, reduces bleeding time, generates fibrin/platelet plug, releases platelet-derived wound healing factors in a sustained manner in the presence of low level collagen, and safe. Development of such a wound healing and hemostatic dressing can be very valuable for short and long-term use in Combat Casualty Care. Pharmaceutical formulation of immobilized L-thyroxine (T4) and globular hexasaccharide in a hydrogel or dressing containing collagen and calcium chloride can be optimized. This novel Wound healing and Hemostatic (WH formulation) treatment in hydrogel or dressing can also include the addition of a microbicidal.

L-thyroxine conjugated to polymer or immobilized on agarose demonstrated potent stimulation of angiogenesis through activation of an adhesion cell surface receptor (integrin αVβ3) leading to activation of an intracellular signaling event, which in turn leads to up-regulation of various growth factor productions. Additionally, immobilized T4 induced epithelial, fibroblast, and keratinocyte cell migration. Immobilized T4, but not T3 or other analogs, enhanced collagen-induced platelet aggregation and secretion, which would promote formation of the subject's own platelet plug. Furthermore, immobilized T4 also promotes white blood cell migration, which could be critical for fighting infection. Hence, immobilized T4 can help the body make more of a compound used to regenerate damaged blood vessels, and it also increased the amount of white blood cells that makes free radicals in the wound site. Free radicals help clear potentially pathogenic bacteria from a wound.

Thus, T4 or T4-agarose (10-100 nM), but not T3, DIPTA, or GC-1, is effective in enhancing platelet aggregation and secretion (de-granulation). Accordingly, T4 (or analogs and polymeric conjugations thereof, e.g., T4-agarose), in combination with 10 mM calcium chloride, and with or without collagen, is preferred for wound healing. See FIGS. **23**A-E.

Thromboelastography

Thromboelastography (TEG) has been used in various hospital settings since its development by Hartert in 1948. The principle of TEG is based on the measurement of the physical viscoelastic characteristics of blood clot. Clot formation was monitored at 37° C. in an oscillating plastic cylindrical cuvette ("cup") and a coaxially suspended stationary piston ("pin") with a 1 mm clearance between the surfaces, using a computerized Thrombelastograph (TEG Model 3000, Haemoscope, Skokie, Ill.). The cup oscillates in either direction every 4.5 seconds, with a 1 second mid-cycle stationary period; resulting in a frequency of 0.1 Hz and a maximal shear rate of 0.1 per second. The pin is suspended by a torsion wire that acts as a torque transducer. With clot formation, fibrin fibrils physically link the cup to the pin and the rotation of the cup as affected by the viscoelasticity of the clot (Transmitted to the pin) is displayed on-line using an IBM-compatible personal computer and customized software (Haemoscope Corp., Skokie, Ill.). The torque experienced by the pin (relative to the cup's oscillation) is plotted as a function of time.

TEG assesses coagulation by measuring various parameters such as the time latency for the initial initiation of the clot (R), the time to initiation of a fixed clot firmness (k) of about 20 mm amplitude, the kinetic of clot development as measured by the angle (α), and the maximum amplitude of the clot (MA). The parameter A measures the width of the tracing at any point of the MA. Amplitude A in mm is a function of clot strength or elasticity. The amplitude on the TEG tracing is a measure of the rigidity of the clot; the peak strength or the shear elastic modulus attained by the clot, G, is a function of clot rigidity and can be calculated from the maximal amplitude (MA) of the TEG tracing.

The following parameters were measured from the TEG tracing:

R, the reaction time (gelation time) represents the latent period before the establishment of a 3-dimensional fibrin gel network (with measurable rigidity of about 2 mm amplitude).

Maximum Amplitude (MA, in mm), is the peak rigidity manifested by the clot.

Shear elastic modulus or clot strength (G, dynes/cm^2) is defined by: G=(5000A)/(100-A).

Blood clot firmness is an important parameter for in vivo thrombosis and hemostasis because the clot must stand the shear stress at the site of vascular injury. TEG can assess the efficacy of different pharmacological interventions on various factors (coagulation activation, thrombin generation, fibrin formation, platelet activation, platelet-fibrin interaction, and fibrin polymerization) involved in clot formation and retraction. The effect of endotoxin (0.63 ug), Xa (0.25 μM), thrombin (0.3 mU), and TF (25 ng) on the different clot parameters measured by computerized TEG in human whole blood is shown in Table 3.

Blood Sampling: Blood was drawn from consenting volunteers under a protocol approved by the Human Investigations Committee of William Beaumont Hospital. Using the two syringe method, samples were drawn through a 21 gauge butterfly needle and the initial 3 ml blood was discarded. Whole blood (WB) was collected into siliconized Vacutainer tubes (Becton Dickinson, Rutherford, N.J. containing 3.8% trisodium citrate such that a ratio of citrate whole blood of 1:9 (v/v) was maintained. TEG was performed within 3 hrs of blood collection. Calcium was added back at 1-2.5 mM followed by the addition of the different stimulus. Calcium chloride by itself at the concentration used showed only a minimal effect on clot formation and clot strength.

Clot formation is initiated by thrombin-induced cleavage of Fibrinopeptide A from fibrinogen. The resultant fibrin monomers spontaneously polymerize to form fibril strands that undergo linear extension, branching, and lateral association leading to the formation of a three-dimensional network of fibrin fibers. A unique property of network structures is that they behave as rigid elastic solids, capable of resisting deforming shear stress. This resistance to deformation can be measured by elastic modulus-an index of clot strength. Unlike conventional coagulation tests (like the prothrombin time and partial thromboplastin time) that are based only on the time to the onset of clot formation, TEG allows acquisition of quantitative information allowing measurement of the maximal strength attained by clots. Via the GPIIb/IIIa receptor, platelets bind fibrin(ogen) and modulate the viscoelastic properties of clots. Our results demonstrated that clot strength in TF-TEG is clearly a function of platelet concentration and platelets augmented clot strength 8 fold under shear. Different

platelet GPIIb/IIIa antagonists (class I versus class II) behaved with distinct efficacy in inhibiting platelet-fibrin mediated clot strength using TF-TEG under shear.

Statistical Analysis

Data are expressed as mean ±SEM. Data were analyzed by either paired or group analysis using the Student t test or ANOVA when applicable; differences were considered significant at $P<0.05$.

TABLE 3

Effect of Calcium Chloride versus Tissue Factor on clot dynamics in citrated human whole blood using TEG

TEG Parameters	25 ng TF + 2.25 mM Ca^{+2} (Mean ± SEM)	10 mM Ca^{+2} (Mean ± SEM)
r (min)	29.7 ± 2.3	14.5 ± 2.5*
K (min)	5.8 ± 1.0	7.0 ± 0.7
α (angle)	45.0 ± 2.6	47.3 ± 2.7
MA (mm)	58.2 ± 1.7	56.5 ± 2.2

Data represent mean ± SEM, n = 4,
*P < 0.01.

Platelet aggregation and de-granulation in whole blood using Impedance Technique: The Model 560 Whole-Blood Aggregometer and the associated Aggro-Link Software from the Chrono-Log Corporation were used in this study. Two electrodes are placed in diluted blood and an electrical impulse is sent from one to the other. As the platelets aggregate around the electrodes, the Chrono-Log measures the impedance of the electrical signal in ohms of resistance.

Blood Sampling

Whole blood was drawn daily from healthy donors between the ages of 17 and 21 into 4.5 milliliter Vacutainer vials with 3.8% buffered sodium citrate (Becton Dickinson, Rutherford, N.J.). The blood was kept on a rocker to extend the life of the platelets, and experiments were done within 5 hours of phlebotomy.

Procedure: For the control, 500 microliters of whole blood, 500 microliters of 0.9% saline, and a magnetic stir bar were mixed into a cuvette, and heated for five minutes to 37 degrees Celsius. Sub-threshold aggregation was induced with 5 microliters of 1-2 μg/ml Collagen, which the Aggregometer measured for 6-7 minutes. The effects of T4, T4-agarose versus T3 and other thyroid hormone analogs on collagen-induced aggregation and secretion were tested. Ingerman-Wojenski C, Smith J B, Silver M J. Evaluation of electrical aggregometry: comparison with optical aggregometry, secretion of ATP, and accumulation of radiolabeled platelets. J Lab Clin Med. 1983 January; 101(1):44-52.

Cell Migration Assay

Human granulocytes are isolated from shed blood by the method of Mousa et al. and cell migration assays carried out as previously described (Methods In Cell Science, 19 (3): 179-187, 1997, and Methods In Cell Science 19 (3): 189-195, 1997). Briefly, a neuroprobe 96 well disposable chemotaxis chamber with an 8 μm pore size will be used. This chamber allow for quantitation of cellular migration towards a gradient of chemokine, cytokine or extracellular matrix proteins. Cell suspension (45 μl of 2×10^6) will be added to a polypropylene plate containing 5 μl of test agents such as flavanoids or thyroid hormone derivatives and incubated for 10 minutes at 22° C. IL8 (0.1-100 ng) with or without T3/T4 (33 μL) at 0.001-0.1 μM will be added to the lower wells of a disposable chemotaxis chamber, then assemble the chamber using the pre-framed filter. Add 25 μl of cell/test agent suspension to the upper filter wells then incubate overnight (22 hours at 37° C., 5% CO2) in a humidified cell culture incubator. After the overnight incubation, non-migrated cells and excess media will be gently removed using a 12 channel pipette and a cell scraper. The filters will then washed twice in phosphate buffered saline (PBS) and fixed with 1% formaldehyde in PBS buffer. Membranes of migrated cells will be permeated with Triton X-100 (0.2%) then washed 2-3 times with PBS. The actin filaments of migrated cells will be stained with Rhodamine phalloidin (12.8 IU/ml) for 30 minutes (22° C.). Rhodamine phalloidin will be made fresh weekly and reused for up to 3 days, when stored protected from light at 4° C. Chemotaxis will be quantitatively determined by fluorescence detection using a Cytofluor II micro-filter fluorimeter (530 excitation/590 emission). All cell treatments and subsequent washings will be carried out using a uniquely designed treatment/wash station (Methods In Cell Science, 19 (3): 179-187, 1997). This technique will allow for accurate quantitation of cell migration and provide reproducible results with minimal inter and intra assay variability.

Cellular Migration Assays

These assays were performed using a Neuroprobe 96 well disposable chemotaxis chamber with an 8 μm pore size. This chamber allowed for quantitation of cellular migration towards a gradient of either vitronectin or osteopontin. Cultured cells were removed following a standardized method using EDTA/Trypsin (0.01%/0.025%). Following removal, the cells were washed twice and resuspended (2×10^6/ml) in EBM (Endothelial cell basal media, Clonetics Inc.). Add either vitronectin or osteopontin (33 μl) at 0.0125-100 μg/ml to the lower wells of a disposable chemotaxis chamber, and then assemble using the preframed filter. The cell suspension (45 μl) was added to a polypropylene plate containing 5 μl of test agent at different concentrations and incubated for 10 minutes at 22° C. Add 25 μl of cell/test agent suspension to the upper filter wells then incubate overnight (22 hours at 37° C.) in a humidified cell culture incubator. After the overnight incubation, non-migrated cells and excess media were gently removed using a 12 channel pipette and a cell scraper. The filters were then washed twice in PBS (no Ca^{+2} or Mg^{+2}) and fixed with 1% formaldehyde. Membranes of migrated cells were permeated with Triton X-100 (0.2%) then washed 2-3 times with PBS. The actin filaments of migrated cells were stained with rhodamine phalloidin (12.8 IU/ml) for 30 minutes (22° C.). Rhodamine phalloidin was made fresh weekly and reused for up to 3 days, when stored protected from light at 4° C. Chemotaxis was quantitatively determined by fluorescence detection using a Cytofluor II (530 excitation/590 emission). All cell treatment and subsequent washings were carried out using a uniquely designed treatment/wash station. This station consisted of six individual reagent units each with a 30 ml volume capacity. Individual units were filled with one of the following reagents: PBS, formaldehyde, Triton X-100, or rhodamine-phalloidin. Using this technique, filters were gently dipped into the appropriate solution, thus minimizing migrated cell loss. This technique allowed for maximum quantitation of cell migration and provided reproducible results with minimal inter and intra assay variability (1, 2).

Migration toward the extracellular Matrix Protein Vitronectin

Treatments (Fluorescence Units) + SD	Mean EC Migration
A. Non-Specific Migration No Matrix in LC	270 ± 20

-continued

Migration toward the extracellular Matrix Protein Vitronectin	
Treatments (Fluorescence Units) + SD	Mean EC Migration
B. Vitronectin (25 ug) in LC	6,116 ± 185
C. T3 (0.1 uM) UC/ Vitronectin (25 ug) in LC	22,016 ± 385
D. T4 (0.1 uM) UC/ Vitronectin (25 ug) in LC	13,083 ± 276
C + XT199 (10 uM)	4,550 ± 225
D + XT199 (10 uM)	3,890 ± 420
C + PD (0.8 ug)	7,555 ± 320
D + PD (0.8 ug)	6,965 ± 390

LC = Lower Chamber,

UC = Upper chamber

Similar data were obtained with other potent and specific avb3 antagonists such as LM609 and SM256

Example 9B

In Vitro Human Epithelial and Fibroblast Wound Healing

The in vitro 2-dimensional wound healing method is as described in Mohamed S, Nadijcka D, Hanson, V. Wound healing properties of cimetidine in vitro. Drug Intell Clin Pharm 20: 973-975; 1986, incorporated herein by reference in its entirety. Additionally, a 3-dimensional wound healing method already established in our Laboratory will be utilized in this study (see below). Data show potent stimulation of wound healing by thyroid hormone.

In Vitro 3D Wound Healing Assay of Human Dermal Fibroblast Cells

Step 1: Prepare Contracted Collagen Gels:

1) Coat 24-well plate with 350 ul 2% BSA at RT for 2 hr,
2) 80% confluent NHDF (normal human dermal fibroblast cells, Passage 5-9) are trypsinized and neutralized with growth medium, centrifuge and wash once with PBS
3) Prepare collagen-cell mixture, mix gently and always on ice:

Stock solution	Final Concentration
5 × DMEC	1 × DMEM
3 mg/ml vitrogen	2 mg/ml
ddH2O	optimal
NHDF	2 × 10~5 cells/ml
FBS	1%

4) Aspire 2% BSA from 24 well plate, add collagen-cell mixture 350 ul/well, and incubate the plate in 37° C. CO_2 incubator.
5) After 1 hr, add DMEM+5% FBS medium 0.5 ml/well, use a 10 ul tip Detach the collagen gel from the edge of each well, then incubate for 2 days. The fibroblast cells will contract the collagen gel

Step 2: Prepare 3D Fibrin Wound Clot and Embed Wounded Collagen Culture

1) Prepare fibrinogen solution (1 mg/ml) with or without testing regents. 350 ul fibrinogen solution for each well in eppendorf tube.

Stock solution	Final Concentration
5 × DMEC	1 × DMEM
Fibrinogen	1 mg/ml
ddH2O	optimal
testing regents	optimal concentration
FBS	1% or 5%

2) Cut each contracted collagen gel from middle with scissors. Wash the gel with PBS and transfer the gel to the center of each well of 24 well plate
3) Add 1.5 ul of human thrombin (0.25 U/ul) to each tube, mix well and then add the solution around the collagen gel, the solution will polymerize in 10 mins.

After 20 mins, add DMEM+1% (or 5%) FBS with or without testing agent, 450 ul/well and incubate the plate in 37° C. CO_2 incubator for up to 5 days. Take pictures on each day.

In Vivo Wound Healing in Diabetic Rats

Using an acute incision wound model in diabetic rats, the effects of thyroid hormone analogs and its conjugated forms are tested. The rate of wound closure, breaking strength analyses and histology are performed periodically on days 3-21.

Methods

Animals (Mice and Rats) in the study are given two small puncture wounds—WH is applied to one of the wounds, and the other was covered with saline solution as a control. Otherwise, the wounds are left to heal naturally.

The animals are euthanised five days after they are wounded. A small area of skin—1 to 1.5 millimetres—is excised from the edges of the treated and untreated wounds.

Wound closure and time to wound closure is determined. Additionally, the levels of tenascin, a protein that helps build connective tissue, in the granulation tissue of the wounds is determined. The quality of the granulation tissue (i.e. rough, pinkish tissue that normally forms as a wound heals, new capillaries and connective tissue) is also determined.

Materials and Methods

Chronic granulating wounds are prepared by methods well known in the art. Male Sprague Dawley rats weighing 300 to 350 grams are acclimatized for a week in our facility prior to use. Under intraperitoneal Nembutal anesthesia (35 mg/kg), the rat dorsum is shaved and depilated. Animals are individually caged and given food and water ad libitum. All experiments were conducted in accordance with the Animal Care and Use Committee guidelines of the Department of Veterans Affairs Medical Center, Albany, N.Y.

Histological characterization of this wound with comparison to a human chronic granulating wound had previously been performed. Sixty four rats are then divided into eight treatment groups (n=8/group). Animals are treated with topical application of vehicle (vehicle controls) on days 5, 9, 12, 15, and 18. The vehicle control can be either agarose (Group 1) or the polymeric form (Group 2) that will be used in conjugation of L-thyroxine. Wounds treated with T4-agarose (Groups 3-5) or T4-polymer (Groups 6-8) at 1, 10, 100 $\mu g/cm^2$ in the presence of 10 μg globular hexasaccharide, 10 μg collagen, and 10 mM calcium chloride to be applied topically on days 5, 9, 12, 15, and 18. All wounds are left exposed. Every 48 hours the outlines of the wounds can be traced onto acetate sheets, and area calculations can be performed using computerized digital planimetry.

Three full-thickness, transverse strips of granulation tissue are then harvested from the cephalad, middle, and caudal ends of the wounds on day 19 and fixed in 10-percent buffered

formalin. Transverse sections (5 μm) are taken from each specimen and stained with hematoxylin and eosin. The thickness of the granulation tissue can be estimated with an ocular micrometer at low power. High-powered fields are examined immediately below the superficial inflammatory layer of the granulation tissue. From each strip of granulation tissue five adjacent high-powered fields can be photographed and coded. Enlarged prints of these exposures are then used for histometric analysis in a blinded fashion. Fibroblasts, "round" cells (macrophages, lymphocytes, and neutrophils), and capillaries are counted. In addition the cellularity of each section is graded for cellularity on a scale of 1 (reduced cell counts) to 5 (highly cellular).

Statistical Analysis

Serial area measurements were plotted against time. For each animal's data a Gompertz equation will be fitted (typical r 2=0.85). Using this curve the wound half-life can be estimated. Comparison between groups is performed using life table analysis and the Wilcoxon rank test. These statistical analyses are performed using the SAS (SAS/STAT Guide for Personal Computers, Version 6 Edition, Cary, N.C., 1987, p 1028) and BMDP (BMDP Statistical Software Manual, Los Angeles, BMDP Statistical Software, Inc. 1988) packages on a personal computer.

Cell counts for the different treatment groups are pooled and analyzed using a one-way analysis of variance. Post-hoc analyses of differences between groups can be carried out using Tukey's test (all pairwise multiple-comparison test) with $p<0.05$ considered significant. Sigma Stat statistical software (Jandel Scientific, Corte Madera, Calif.) will be used for data analysis.

Example 10

Rodent Model of Myocardial Infarction

The coronary artery ligation model of myocardial infarction is used to investigate cardiac function in rats. The rat is initially anesthetized with xylazine and ketamine, and after appropriate anesthesia is obtained, the trachea is intubated and positive pressure ventilation is initiated. The animal is placed supine with its extremities loosely taped and a median sternotomy is performed. The heart is gently exteriorized and a 6-O suture is firmly tied around the left anterior descending coronary artery. The heart is rapidly replaced in the chest and the thoracotomy incision is closed with a 3-O purse string suture followed by skin closure with interrupted sutures or surgical clips. Animals are placed on a temperature regulated heating pad and closely observed during recovery. Supplemental oxygen and cardiopulmonary resuscitation are administered if necessary. After recovery, the rat is returned to the animal care facility. Such coronary artery ligation in the rat produces large anterior wall myocardial infarctions. The 48 hr. mortality for this procedure can be as high as 50%, and there is variability in the size of the infarct produced by this procedure. Based on these considerations, and prior experience, to obtain 16-20 rats with large infarcts so that the two models of thyroid hormone delivery discussed below can be compared, approximately 400 rats are required.

These experiments are designed to show that systemic administration of thyroid hormone either before or after coronary artery ligation leads to beneficial effects in intact animals, including the extent of hemodynamic abnormalities assessed by echocardiography and hemodynamic measurements, and reduction of infarct size. Outcome measurements are proposed at three weeks post-infarction. Although some rats may have no infarction, or only a small infarction is produced, these rats can be identified by normal echocardiograms and normal hemodynamics (LV end-diastolic pressure<8 mm Hg).

Thyroid Hormone Delivery

There are two delivery approaches. In the first, thyroid hormone is directly injected into the peri-infarct myocardium. As the demarcation between normal and ischemic myocardium is easily identified during the acute open chest occlusion, this approach provides sufficient delivery of hormone to detect angiogenic effects.

Although the first model is useful in patients undergoing coronary artery bypass surgery, and constitutes proof of principle that one local injection induces angiogenesis, a broader approach using a second model can also be used. In the second model, a catheter retrograde is placed into the left ventricle via a carotid artery in the anesthetized rat prior to inducing myocardial infarction. Alternatively, a direct needle puncture of the aorta, just above the aortic valve, is performed. The intracoronary injection of the thyroid hormone is then simulated by abruptly occluding the aorta above the origin of the coronary vessels for several seconds, thereby producing isovolumic contractions. Thyroid hormone is then injected into the left ventricle or aorta immediately after aortic constriction. The resulting isovolumic contractions propel blood down the coronary vessels perfusing the entire myocardium with thyroid hormone. This procedure can be done as many times as necessary to achieve effectiveness. The number of injections depends on the doses used and the formation of new blood vessels.

Echocardiography

A method for obtaining 2-D and M-mode echocardiograms in unanesthetized rats has been developed. Left ventricular dimensions, function, wall thickness and wall motion can be reproducibly and reliably measured. The measurement are carried out in a blinded fashion to eliminate bias with respect to thyroid hormone administration.

Hemodynamics

Hemodynamic measurements are used to determine the degree of left ventricular impairment. Rats are anesthetized with isoflurane. Through an incision along the right anterior neck, the right carotid artery and the right jugular vein are isolated and cannulated with a pressure transducing catheter (Millar, SPR-612, 1.2 Fr). The following measurements are then made: heart rate, systolic and diastolic BP, mean arterial pressure, left ventricular systolic and end-diastolic pressure, and + and −dP/dt. Of particular utility are measurements of left ventricular end-diastolic pressure, progressive elevation of which correlates with the degree of myocardial damage.

Infarct Size

Rats are sacrificed for measurement of infarct size using TTC methodology.

Morphometry

Microvessel density [microvessels/mm^2] will be measured in the infarct area, peri-infarct area, and in the spared myocardium opposing the infarction, usually the posterior wall. From each rat, 7-10 microscopic high power fields [×400] with transversely sectioned myocytes will be digitally recorded using Image Analysis software. Microvessels will be counted by a blinded investigator. The microcirculation will be defined as vessels beyond third order arterioles with a diameter of 150 micrometers or less, supplying tissue between arterioles and venules. To correct for differences in left ventricular hypertrophy, microvessel density will be divided by LV weight corrected for body weight. Myocardium from sham operated rats will serves as controls.

Example 11

Effects of the αVβ3 Antagonists on the Pro-Angiogenesis Effect of T4 or FGF2

The αVβ3 inhibitor LM609 totally inhibited both FGF2 or T4-induced pro-angiogenic effects in the CAM model at 10 micrograms (FIG. **16**).

Example 12

Inhibition of Cancer-Related New Blood Vessel Growth

A protocol disclosed in J. Bennett, Proc Natl Acad Sci USA 99:2211-2215, 2002, is used for the administration of tetraiodothyroacetic (Tetrac) to SCID mice that have received implants of human breast cancer cells (MCF-7). Tetrac is provided in drinking water to raise the circulating level of the hormone analog in the mouse model to 10^{-6} M. The endpoint is the inhibitory action of tetrac on angiogenesis about the implanted tumors.

Example 13

Pro-Angiogenesis Promoting Effect of Thyroid Hormone and Analogs Thereof at Subthreshold Levels of VEGF and FGF2 in an In Vitro Three-Dimensional Micro-Vascular Endothelial Sprouting Model

Either T_3, T_4, T_4-agarose, or fibroblast growth factor 2 (FGF2) plus vascular endothelial growth factor (VEGF) produced a comparable pro-angiogenesis effect in the in vitro three-dimensional micro-vascular endothelial sprouting model. The pro-angiogenesis effect of the thyroid hormone analogs were blocked by PD 98059, an inhibitor of the mitogen-activated protein kinase (MAPK; ERK1/2) signal transduction cascade. Additionally, a specific αVβ3 integrin antagonist (XT199) inhibited the pro-angiogenesis effect of either thyroid hormone analogs or T4-agarose. Data also demonstrated that the thyroid hormone antagonist Tetrac inhibits the thyroid analog's pro-angiogenesis response. Thus, those thyroid hormone analogs tested are pro-angiogenic, an action that is initiated at the plasma membrane and involves αVβ3 integrin receptors, and is MAPK-dependent.

The present invention describes a pro-angiogenesis promoting effect of T_3, T_4, or T_4-agarose at sub-threshold levels of VEGF and FGF2 in an in vitro three-dimensional micro-vascular endothelial sprouting model. The invention also provides evidence that the hormone effect is initiated at the endothelial cell plasma membrane and is mediated by activation of the αVβ3 integrin and ERK1/2 signal transduction pathway.

Enhancement by T_3, T_4, or T_4 agarose of the angiogenesis activity of low concentrations of VEGF and FGF2 in the three-dimensional sprouting assay was demonstrated. Either T_3, T_4 at 10^{-7}-10^{-8}M, or T_4-agarose at 10^{-7} M total hormone concentration was comparable in pro-angiogenesis activity to the maximal concentrations of VEGF and FGF2 effect in this in vitro model. Although new blood vessel growth in the rat heart has been reported to occur concomitantly with induction of myocardial hypertrophy by a high dose of T_4, thyroid hormone has not been regarded as an angiogenic factor. The present example establishes that the hormone in physiologic concentrations is pro-angiogenic in a setting other than the heart.

T_4-agarose reproduced the effects of T_4, and this derivative of thyroid hormone is thought not to gain entry to the cell interior; it has been used in our laboratory to examine models of hormone action for possible cell surface-initiated actions of iodothyronines. Further, experiments carried out with T_4 and tetrac also supported the conclusion that the action of T_4 in this model was initiated at the plasma membrane. Tetrac blocks membrane-initiated effects of T_4.

Since thyroid hormone non-genomically activates the MAPK (ERK1/2) signal transduction pathway, the action of the hormone on angiogenesis can be MAPK-mediated. When added to the CAM model, an inhibitor of the MAPK cascade, PD 98059, inhibited the pro-angiogenic action of T_4. While this result was consistent with an action on transduction of the thyroid hormone signal upstream of an effect of T_4 on FGF2 elaboration, it is known that FGF2 also acts via an MAPK-dependent mechanism. T_4 and FGF2 individually cause phosphorylation and nuclear translocation of ERK1/2 in endothelial cells and, when used in sub-maximal doses, combine to enhance ERK1/2 activation further. To examine the possibility that the only MAPK-dependent component of hormonal stimulation of angiogenesis related exclusively to the action of FGF2 on vessel growth, cellular release of FGF2 in response to T_4 in the presence of PD 98059 was measured. The latter agent blocked the hormone-induced increase in growth factor concentration and indicated that MAPK activation was involved in the action of T_4 on FGF2 release from endothelial cells, as well as the consequent effect of FGF2 on angiogenesis.

Effect of Thyroid Hormone on Angiogenesis

Either T_4, T_3, or T4-agarose at 0.01-0.1 μM resulted in significant ($P<0.01$) stimulation of angiogenesis (Table 4). This is shown to be comparable to the pro-angiogenesis efficacy of FGF2 (50 ng/ml) plus VEGF (25 ng/ml).

TABLE 4

In Vitro Pro-angiogenesis Effect of Growth Factors, Thyroid Hormone, and Analogs in the Three-Dimensional Human Micro-vascular Endothelial Sprouting Assay

Treatment Groups	Mean Tube Vessel Length (mm) ± SD
Control	0.76 ± 0.08
FGF2 (25 ng) + VEGF (50 ng)	2.34 ± 0.25*
T3 (20 ng)	1.88 ± 0.21*
T4 (23 ng)	1.65 ± 0.15*
T4-agarose (23 ng)	1.78 ± 0.20*

Data (means ± SD) were obtained from 3 experiments. Cells were pre-treated with Subthreshold level of FGF2 (1.25 ng/ml) + VEGF(2.5 ng/ml). Data represent mean ± SD, n = 3, *P < 0.01 by ANOVA, comparing treated to control.

Effects of Tetrac on Thyroid Pro-Angiogenesis Action

T_3 stimulates cellular signal transduction pathways initiated at the plasma membrane. These pro-angiogenesis actions are blocked by a deaminated iodothyronine analogue, tetrac, which is known to inhibit binding of T_4 to plasma membranes. The addition of tetrac (0.1 μM) inhibited the pro-angiogenesis action of either T_3, T_4, or T_4-agarose (Tables 5-7). This is shown by the inhibition of number of micro-vascular endothelial cell migration and vessel length (Table 5-7).

Role of the ERK1/2 Signal Transduction Pathway in Stimulation of Angiogenesis by Thyroid Hormone

Parallel studies of ERK1/2 inhibition were carried out in the three-dimensional micro-vascular sprouting assays. Thyroid hormone and analog at 0.01-0.1 μM caused significant increase in tube length and number of migrating cells, an effect that was significantly ($P<0.01$) blocked by PD 98059

(Tables 5-7). This is shown by the inhibition of number micro-vascular endothelial cell migration and vessel length (Table 5-7).

Role of the Integrin αVβ3 in Stimulation of Angiogenesis by Thyroid Hormone

Either T_3, T_4, or T_4-agarose at 0.01-0.1 μm-mediated pro-angiogenesis in the presence of sub-threshold levels of VEGF and FGF2 was significantly ($P<0.01$) blocked by the αVβ3 integrin antagonist XT199 (Tables 5-7). This is shown by the inhibition of number of micro-vascular endothelial cell migration and vessel length (Table 5-7).

Thus, the pro-angiogenesis effect of thyroid hormone and its analogs begins at the plasma membrane αVβ3 integrin and involves activation of the ERK1/2.

TABLE 5

Pro-angiogenesis Mechanisms of the Thyroid Hormone T_3 in the Three-Dimensional Human Micro-vascular Endothelial Sprouting Assay

HDMEC treatment	Mean number of Migrated cells ± SD	Mean vessel Length (mm) ± SD
Control	88 ± 14	0.47 ± 0.06
T_3 (0.1 uM)	188 ± 15*	0.91 ± 0.04*
T_3 (0.1 uM) + PD98059 (3 ug)	124 ± 29	0.48 ± 0.09
T_3 (0.1 uM) + XT199 (2 ug)	118 ± 18	0.47 ± 0.04
T_3 (0.1 uM) + tetrac (0.15 ug)	104 ± 15	0.58 ± 0.07

Human dermal micro-vascular endothelial cells (HDMVC) were used. Cells were pretreated with FGF2 (1.25 ng/ml) + VEGF (2.5 ng/ml). Images were taken at 4 and 10X, day 3. Data represent mean ± SD, n = 3,
*$P < 0.01$.

TABLE 6

Pro-angiogenesis Mechanisms of the Thyroid Hormone T_4 in the Three-Dimensional Human Micro-vascular Endothelial Sprouting Assay

HDMEC treatment	Mean number of Migrated cells ± SD	Mean Vessel Length (mm) ± SD
Control	88 ± 14	0.47 ± 0.06
T_4 (0.1 uM)	182 ± 11*	1.16 ± 0.21*
T_4 (0.1 uM) + PD98059 (3 ug)	110 ± 21	0.53 ± 0.13
T_4 (0.1 uM) + XT199 (2 ug)	102 ± 13	0.53 ± 0.05
T_4 (0.1 uM) + Tetrac (0.15 ug)	85 ± 28	0.47 ± 0.11

Human dermal micro-vascular endothelial cells (HDMVC) were used. Cells were pretreated with FGF2 (1.25 ng/ml) + VEGF (2.5 ng/ml). Images were taken at 4 and 10X, day 3. Data represent mean ± SD, n = 3,
*$P < 0.01$.

TABLE 7

Pro-angiogenesis Mechanisms of the Thyroid Hormone T_4-Agarose in the Three-Dimension Human Micro-vascular Endothelial Sprouting Assay

HDMEC treatment	Mean number of Migrated cells ± SD	Mean Vessel Length (mm) ± SD
Control	88 ± 14	0.47 ± 0.06
T_4-agarose (0.1 uM)	191 ± 13*	0.97 ± 0.08*
T_4-agarose (0.1 uM) + PD98059 (3 ug)	111 ± 8	0.56 ± 0.03
T_4-agarose (0.1 uM) + XT199 (2 ug)	106 ± 5	0.54 ± 0.03
T_4-agarose (0.1 uM) + Tetrac (0.15 ug)	87 ± 14	0.45 ± 0.09

Human dermal micro-vascular endothelial cells (HDMVC) were used. Cells were pretreated with FGF2 (1.25 ng/ml) + VEGF (2.5 ng/ml). Images were taken at 4 and 10X, day 3. Data represent mean ± SD, n = 3,
*$P < 0.01$.

Example 14

In Vitro Model for Evaluating Polymeric Thyroid Analogs Transport Across the Blood-Brain Barrier

Described below is an in vitro method for evaluating the facility with which selected polymeric thyroid analog alone or in combination with nerve growth factor or other neurogenesis factors likely will pass across the blood-brain barrier. A detailed description of the model and protocol are provided by Audus, et al., Ann. N.Y. Acad. Sci. 507: 9-18 (1987), the disclosure of which is incorporated herein by reference.

Briefly, microvessel endothelial cells are isolated from the cerebral gray matter of fresh bovine brains. Brains are obtained from a local slaughter house and transported to the laboratory in ice cold minimum essential medium ("MEM") with antibiotics. Under sterile conditions the large surface blood vessels and meninges are removed using standard dissection procedures. The cortical gray matter is removed by aspiration, then minced into cubes of about 1 mm. The minced gray matter then is incubated with 0.5% dispase (BMB, Indianapolis, Ind.) for 3 hours at 37° C. in a shaking water bath. Following the 3 hour digestion, the mixture is concentrated by centrifugation (1000×g for 10 min.), then resuspended in 13% dextran and centrifuged for 10 min. at 5800×g. Supernatant fat, cell debris and myelin are discarded and the crude microvessel pellet resuspended in 1 mg/ml collagenase/dispase and incubated in a shaking water bath for 5 hours at 37° C. After the 5-hour digestion, the microvessel suspension is applied to a pre-established 50% Percoll gradient and centrifuged for 10 min at 1000×g. The band containing purified endothelial cells (second band from the top of the gradient) is removed and washed two times with culture medium (e.g., 50% MEM/50% F-12 nutrient mix). The cells are frozen (−80° C.) in medium containing 20% DMSO and 10% horse serum for later use.

After isolation, approximately 5×10^5 cells/cm^2 are plated on culture dishes or 5-12 mm pore size polycarbonate filters that are coated with rat collagen and fibronectin. 10-12 days after seeding the cells, cell monolayers are inspected for confluency by microscopy.

Characterization of the morphological, histochemical and biochemical properties of these cells has shown that these cells possess many of the salient features of the blood-brain barrier. These features include: tight intercellular junctions, lack of membrane fenestrations, low levels of pinocytotic activity, and the presence of gamma-glutamyl transpeptidase, alkaline phosphatase, and Factor VIII antigen activities.

The cultured cells can be used in a wide variety of experiments where a model for polarized binding or transport is required. By plating the cells in multi-well plates, receptor and non-receptor binding of both large and small molecules can be conducted. In order to conduct transendothelial cell flux measurements, the cells are grown on porous polycarbonate membrane filters (e.g., from Nucleopore, Pleasanton, Calif.). Large pore size filters (5-12 mm) are used to avoid the possibility of the filter becoming the rate-limiting barrier to molecular flux. The use of these large-pore filters does not permit cell growth under the filter and allows visual inspection of the cell monolayer.

Once the cells reach confluency, they are placed in a side-by-side diffusion cell apparatus (e.g., from Crown Glass, Sommerville, N.J.). For flux measurements, the donor chamber of the diffusion cell is pulsed with a test substance, then at various times following the pulse, an aliquot is removed from the receiver chamber for analysis. Radioactive or fluorescently-labelled substances permit reliable quantitation of

molecular flux. Monolayer integrity is simultaneously measured by the addition of a non-transportable test substance such as sucrose or insulin and replicates of at least 4 determinations are measured in order to ensure statistical significance.

Example 15

Traumatic Injury Model

The fluid percussion brain injury model was used to assess the ability of polymeric thyroid hormone analogs alone or in combination with nerve growth factors or other neurogenesis factors to restore central nervous system functions following significant traumatic brain injury.

I. Fluid Percussion Brain Injuries Procedure

The animals used in this study were male Sprague-Dawley rats weighing 250-300 grams (Charles River). The basic surgical preparation for the fluid-percussion brain injury has been previously described. Dietrich, et al., Acta Neuropathol. 87: 250-258 (1994) incorporated by reference herein. Briefly, rats were anesthetized with 3% halothane, 30% oxygen, and a balance of nitrous oxide. Tracheal intubation was performed and rats were placed in a stereotaxic frame. A 4.8-mm craniotomy was then made overlying the right parietal cortex, 3.8 mm posterior to bregma and 2.5 mm lateral to the midline. An injury tube was placed over the exposed dura and bonded by adhesive. Dental acrylic was then poured around the injury tube and the injury tube was then plugged with a gelfoam sponge. The scalp was sutured closed and the animal returned to its home case and allowed to recover overnight.

On the next day, fluid-percussion brain injury was produced essentially as described by Dixon, et al., J. Neurosurg. 67: 110-119 (1987) and Clifton, et al., J. Cereb. Blood Flow Metab. 11: 114-121 (1991). The fluid percussion device consisted of a saline-filled Plexiglas cylinder that is fitted with a transducer housing and injury screw adapted for the rat's skull. The metal screw was firmly connected to the plastic injury tube of the intubated anesthetized rat (70% nitrous oxide, 1.5% halothane, and 30% oxygen), and the injury was induced by the descent of a pendulum that strikes the piston. Rats underwent mild-to-moderate head injury, ranging from 1.6 to 1.9 atm. Brain temperature was indirectly monitored with a thermistor probe inserted into the right temporalis muscle and maintained at 37-37.5° C. Rectal temperature was also measured and maintained at 37° C. prior to and throughout the monitoring period.

Example 25:

T4-Stimulated MAPK Activation is Blocked by Inhibitors of Hormone Binding and Binding of Intergrin αVβ3

Nuclear translocation of phosphorylated MAPK (pERK½) was studied in CV-1 cells treated with physiological levels of T4 10^{-7} M total hormone concentration, $10^{31\ 10}$ M free hormone) for 30 min. Consistent with results we have previously reported, T4 induced nuclear accumulation of phosphorylated MAPK in CV-1 cells within 30 min (FIG. **27**). Preincubation of CV-1 cells with the indicated concentrations of αVβ3 antagonists for 16 h reduced the ability T4 to induce MAPK activation and translocation. Application of an RGD peptide at 10^{-8} and 10^{-7} M had a minimal effect on MAPK activation. However, 10^{-6} M RGD peptide inhibited MAPK phosphorylation by 62% compared to control cultures and activation was reduced maximally when 10^{-5} M RGD (85reduction) and 10^{-4} M RGD (87% reduction) were present in the culture media. Addition of the nonspecific RGE peptide to the culture media had no effect on MARK phosphorylation and nuclear translocation following T4 treatment in CV-1 cells.

Tetrac, which prevents the binding of T4 to the plasma membrane, is an effective inhibitor of T4-induced MAPK activation. When present at a concentration of 10^{-6} M with T4, tetrac reduced MAPK phosphorylation and translocation by 86% when compared to cultures treated with T4 alone (FIG. **27**). The inhibition increased to 97% when 10^{-4} M tetrac was added to the culture media for 16 h before the application of T4. Addition of αVβ3 monoclonal antibody LM609 to the culture media 16 h prior to stimulation with T 4 also reduced T4-induced MAPK activation following T4 treatment. Increasing the concentration of antibody in the culture media to 0.1, 1, and 10 μg/ml reduced levels of phosphorylated MAPK found in the nuclear fractions of the cells by 29%, 80%, and 88%, respectively, when compared to cells treated with T4 alone.

CV-1 cells were transiently transfected with siRNA to αV, β3 or both αV and β3 and allowed to recover for 16 h before being placed in serum-free media. Following T4 treatment for 30 min, the cells were harvested and either nuclear protein or RNA was extracted. FIG. **28**A demonstrates the specificity of each siRNA for the target integrin subunit. CV-1 cells transfected with either the αV siRNA or both αV and β3 siRNAs showed decreased αV submit RT-PCR products, but there was no difference in αV mRNA expression when cells were transfected with the siRNA specific for β3, or when exposed to

Behavioral Testing:

Three standard functional/behavioral tests were used to assess sensorimotor and reflex function after brain injury. The tests have been fully described in the literature, including Bederson, et al., (1986) Stroke 17: 472-476; DeRyck, et al., (1992) Brain Res. 573: 44-60; Markgraf, et al., (1992) Brain Res. 575: 238-246; and Alexis, et al., (1995) Stroke 26: 2338-2346.

A. The Forelimb Placing Test

Forelimb placing to three separate stimuli (visual, tactile, and proprioceptive) was measured to assess sensorimotor integration. DeRyck, et al., Brain Res. 573:44-60 (1992). For the visual placing subtest, the animal is held upright by the researcher and brought close to a table top. Normal placing of the limb on the table is scored as "0," delayed placing (<2 sec) is scored as "1," and no or very delayed placing (>2 sec) is scored as "2." Separate scores are obtained first as the animal is brought forward and then again as the animal is brought sideways to the table (maximum score per limb=4; in each case higher numbers denote greater deficits). For the tactile placing subtest, the animal is held so that it cannot see or touch the table top with its whiskers. The dorsal forepaw is touched lightly to the table top as the animal is first brought forward and then brought sideways to the table. Placing each time is scored as above (maximum score per limb=4). For the proprioceptive placing subtest, the animal is brought forward only and greater pressure is applied to the dorsal forepaw; placing is scored as above (maximum score per limb=2). Finally, the ability of animals to place the forelimb in response to whisker stimulation by the tabletop was tested (maximum score per limb=2). Then subscores were added to give the total forelimb placing score per limb (range=0-12).

B. The Beam Balance Test

Beam balance is sensitive to motor cortical insults. This task was used to assess gross vestibulomotor function by requiring a rat to balance steadily on a narrow beam. Feeney,

et al., Science, 217: 855-857 (1982); Goldstein, et al., Behav. Neurosci. 104: 318-325 (1990). The test involved three 60-second training trials 24 hours before surgery to acquire baseline data. The apparatus consisted of a ¾-inch-wide beam, 10 inches in length, suspended 1 ft. above a table top. The rat was positioned on the beam and had to maintain steady posture with all limbs on top of the beam for 60 seconds. The animals' performance was rated with the scale of Clifton, et al., J. Cereb Blood Flow Metab. 11: 1114-121 (1991), which ranges from 1 to 6, with a score of 1 being normal and a score of 6 indicating that the animal was unable to support itself on the beam.

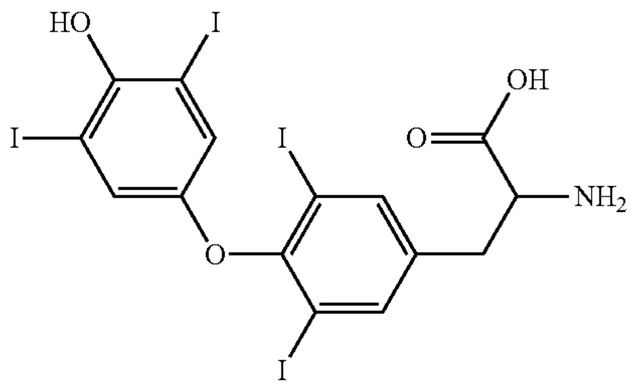
C. The Beam Walking Test

This was a test of sensorimotor integration specifically examining hindlimb function. The testing apparatus and rating procedures were adapted from Feeney, et al., Science, 217: 855-857 (1982). A 1-inch-wide beam, 4 ft. in length, was suspended 3 ft. above the floor in a dimly lit room. At the far end of the beam was a darkened goal box with a narrow entryway. At equal distances along the beam, four 3-inch metal screws were positioned, angling away from the beam's center. A white noise generator and bright light source at the start of the beam motivated the animal to traverse the beam and enter the goal box. Once inside the goal box, the stimuli were terminated. The rat's latency to reach the goal box (in seconds) and hindlimb performance as it traversed the beam (based on a 1 to 7 rating scale) were recorded. A score of 7 indicates normal beam walking with less than 2 foot slips, and a score of 1 indicates that the rat was unable to traverse the beam in less than 80 seconds. Each rat was trained for three days before surgery to acquire the task and to achieve normal performance (a score of 7) on three consecutive trials. Three baseline trials were collected 24 hours before surgery, and three testing trials were recorded daily thereafter. Mean values of latency and score for each day were computed.

Example 16

Thyroid Hormone Analogs

T4



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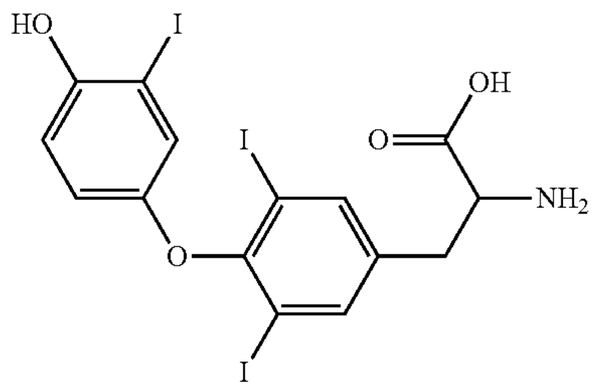
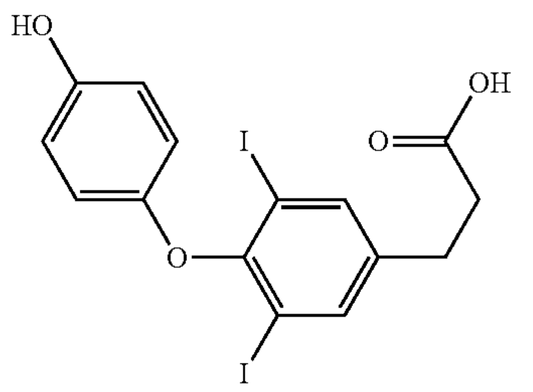
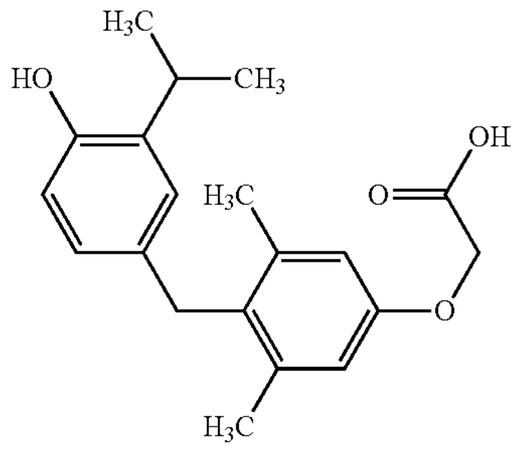
T3



DITPA

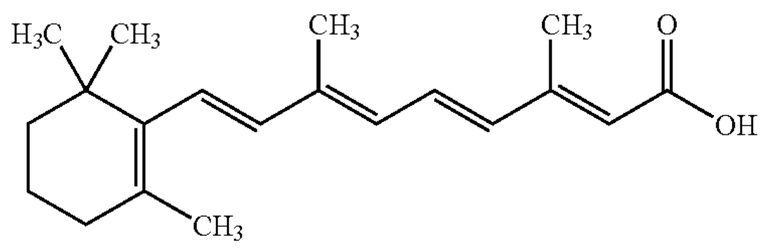


GC-1



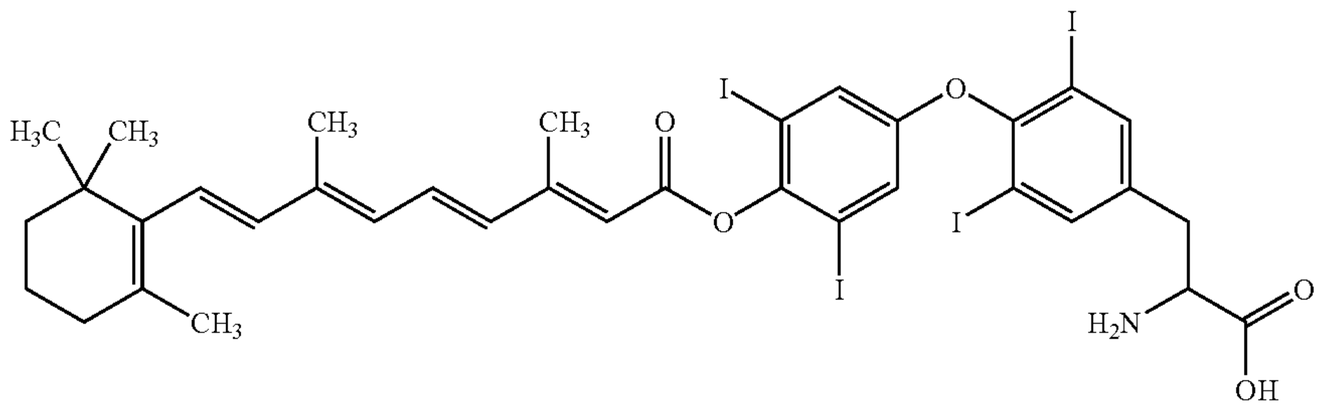
Example 17

Retinoic Acid Analogs



Example 18

Thyroid Hormone Analog Conjugated with Retinoic Acid



T4-Retinoic Acid

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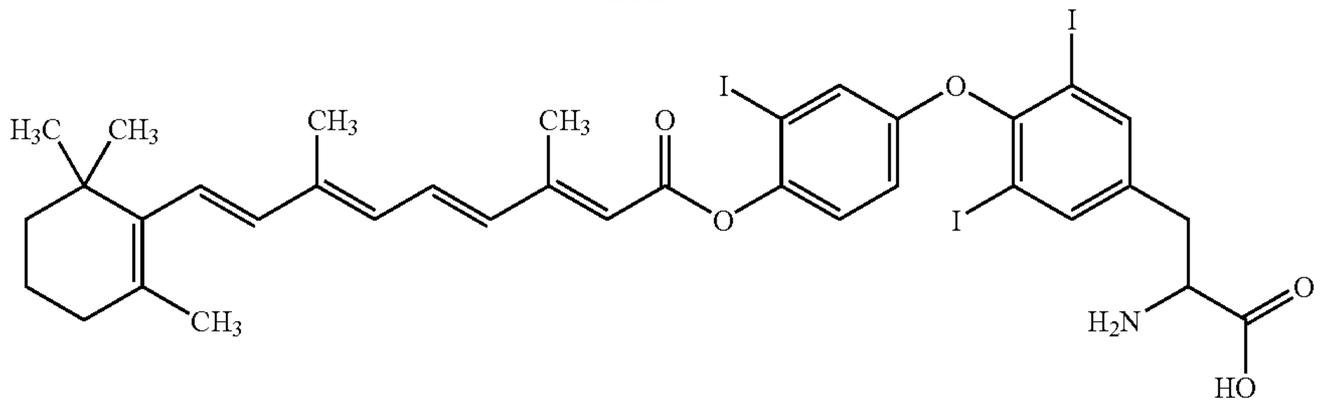
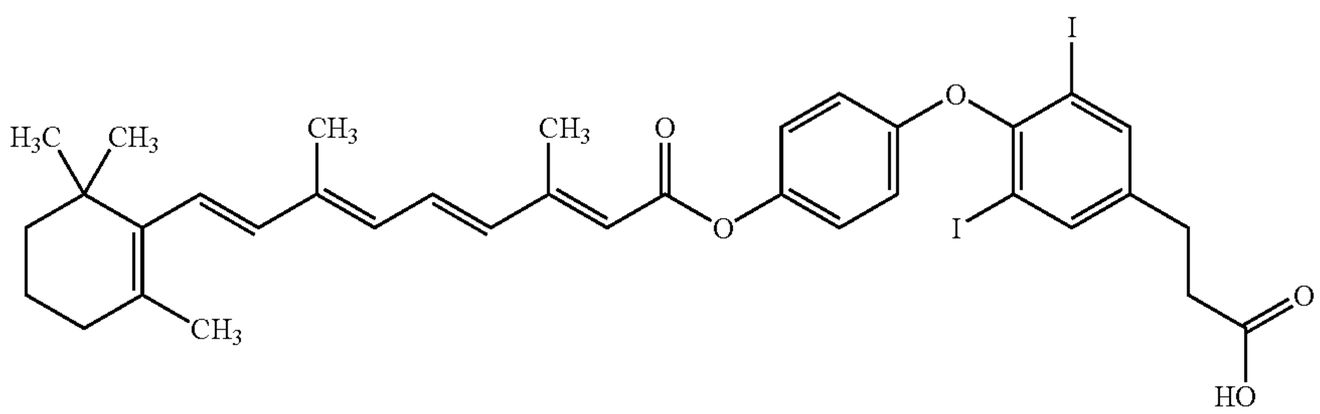
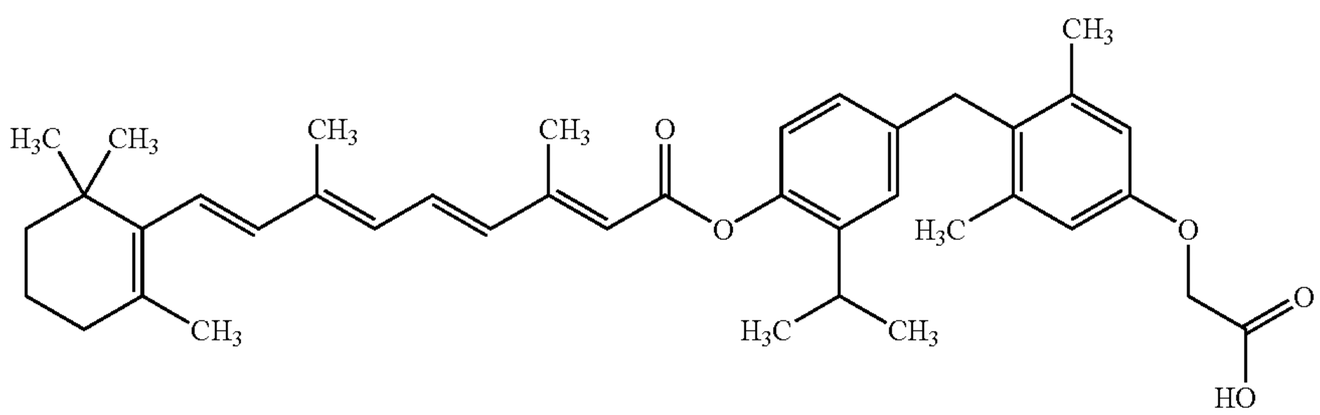


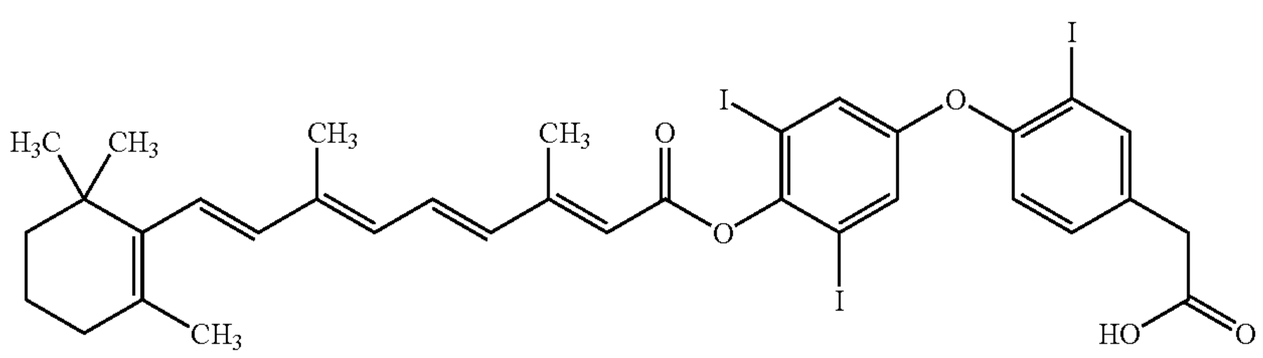
T3-Retinoic Acid



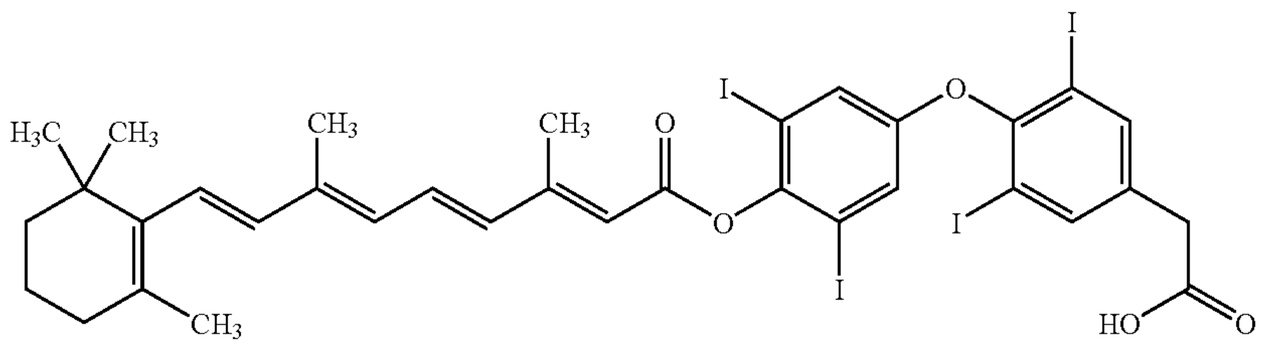
DITPA-Retinoic Acid



GC-1-Retinoic Acid



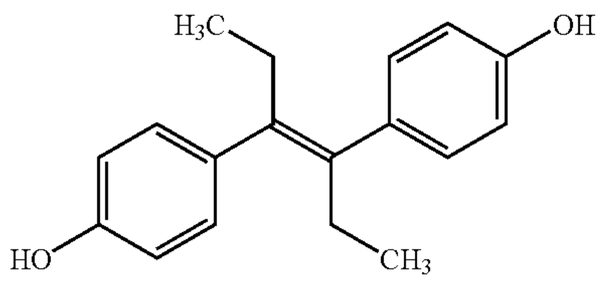
TRIAC-Retinoic Acid



TETRAC-Retinoic Acid

Example 19

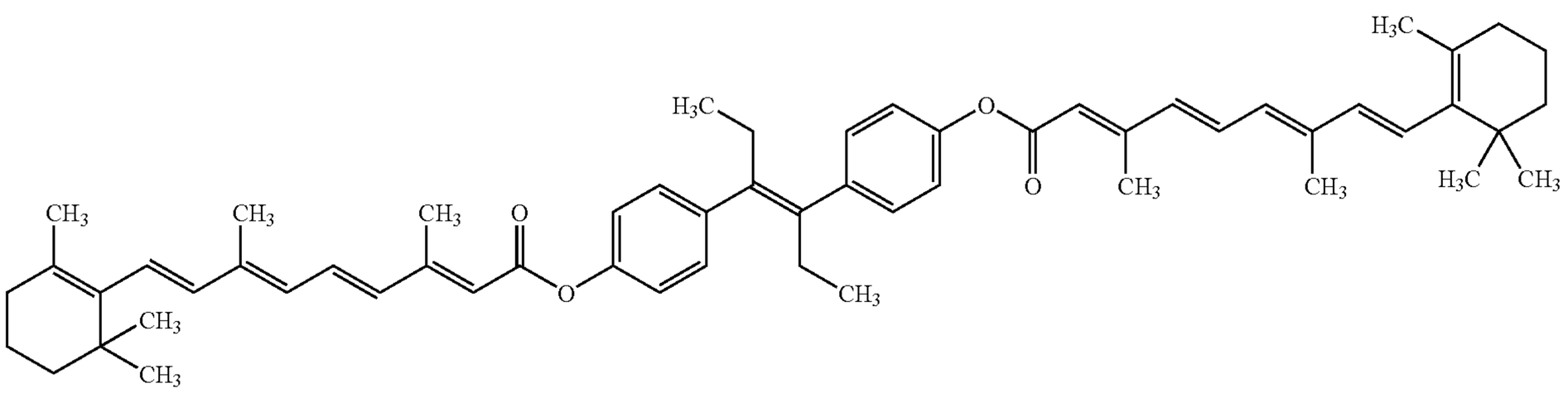
Halogenated Stilbestrol Analogs



Diethylstilbestrol

Example 20

Compositions of T4 Analogs, Halogenated Stilbesterols, and Retinoic Acid



Retinoic Acid-Diethylstilbestrol-Retinoic Acid

Example 21

Preparations of Compounds for Pet-Imaging

In general, the radioactive imaging agents of the present invention (Examples 16-20) are prepared by reacting radioactive 4-halobenzyl derivatives with piperazine derivatives. Preferred are F-18 labeled 4-fluorobenzyl derivatives for PET-imaging. A general method for the preparation of 4-fluoro-.sup.18 F-benzyl halides is described in Iwata et al., Applied Radiation and Isotopes (2000), Vol. 52, pp. 87-92.

Example 22

Preparation of Compounds for SPECT-Imaging

For Single Photon Emission Computed Tomography ("SPECT"), ^{99m}Tc-labeled compounds are preferred. A general synthetic pathway for these compounds starts with non-radioactive analogues of compounds according to Examples 16-20 that are reacted with ^{99m}Tc-binding chelators, e.g. N_2S_2-Chelators. The synthesis of the chelators follows standard procedures, for example, the procedures described in A. Mahmood et al., A N_2S_2-Tetradentate Chelate for Solid-Phase Synthesis: Technetium, Rhenium in Chemistry and Nuclear Medicine (1999), Vol. 5, p. 71, or in Z. P. Zhuang et al., Bioconjugate Chemistry (1999), Vol. 10, p. 159.

One of the chelators is either bound directly to the nitrogen in the —N(R^4)R^5 group of the non-radioactive compounds according to Examples 16-20, or via a linker moiety comprising an alkyl radical having one to ten carbon atoms, wherein the alkyl radical optionally contains one to ten —C(O)— groups, one to ten —C(O)N(R)— groups, one to ten —N(R)C(O)— groups, one to ten —N(R)— groups, one to ten —$N(R)_2$ groups, one to ten hydroxy groups, one to ten —C(O)OR— groups, one to ten oxygen atoms, one to ten sulfur atoms, one to ten nitrogen atoms, one to ten halogen atoms, one to ten aryl groups, and one to ten saturated or unsaturated heterocyclic rings wherein R is hydrogen or alkyl. A preferred linker moiety is —C(O)—CH_2—N(H)—.

Example 23

T4 is a ligand of αVβ3 Integrin

To determine if T4 is a ligand of the αVβ3 integrin, 2 μg of commercially available purified protein was incubated with [^{125}I]T4, and the mixture was run out on a non-denaturing polyacrylamide gel. αVβ3 binds radiolabeled T4 and this interaction was competitively disrupted by unlabeled T4, which was added to αVβ3 prior to the [^{125}I]T4 incubation, in a concentration-dependent manner (FIG. **24**). Addition of unlabeled T4 reduced binding of integrin to the radiolabeled ligand by 13% at a total T4 concentration of 10^{-7} M total (3×10^{-10} M free T4), 58% at 10^{-6} M total (1.6×10^{-9} M free), and inhibition of binding was maximal with 10^{-5} M unlabeled T4. Using non-linear regression, the interaction of αVβ3 with free T4 was determined to have a Kd of 333 pM and an EC_{50} of 371 pM. Unlabeled T3 was less effective in displacing [^{125}I]T4-binding to αVβ3, reducing the signal by 28% at 10^{-4} M total T3.

Example 24

T4 Binding to αVβ3 is Blocked by tetrac, RGD Peptide and Integrin Antibody

We have shown previously that T4-stimulated signaling pathways activated at the cell surface can be inhibited by the iodothyronine analog tetrac, which is shown to prevent binding of T4 to the plasma membrane. In our radioligand-binding assay, while 10^{-8} M tetrac had no effect on [^{125}I]T4-binding to purified αVβ3, the association of T4 and αVβ3 was reduced by 38% in the presence of 10^{-7} M tetrac and by 90% with 10^{-5} M tetrac (FIG. **25**). To determine specificity of the interaction, an RGD peptide, which binds to the extracellular matrix-biding site on αVβ3, and an RGE peptide, which has a glutamic acid residue instead of an aspartic acid residue and thus does not bind αVβ3, were added in an attempt to displace

T4 from binding with the integrin. Application of an RGD peptide, but not an RGE peptide, reduced the interaction of [^{125}I]T4 with αVβ3 in a dose-dependent manner (FIG. **25**).

To further characterize the interaction of T4 with αVβ3, antibodies to αVβ3 or αVβ5 were added to purified αVβ3 prior to addition of [^{125}I]T4. Addition of 1 μg/ml of αVβ3 monoclonal antibody LM609 reduced complex formation between the integrin and T4 by 52%, compared to untreated control samples. Increasing the amount of LM609 to 2 μg, 4 μg, and 8 μg/ml diminished band intensity by 64%, 63% and 81%, respectively (FIG. **26**). Similar results were observed when a different αVβ3 monoclonal antibody, SC7312, was incubated with the integrin. SC7312 reduced the ability of T4 to bind αVβ3 by 20% with 1 μg/ml of antibody present, 46% with 2 μg, 47% with 4 μg, and by 59% when 8 μg/ml of antibody were present. Incubation with monoclonal antibodies to αV and β3, separately, did not affect [^{125}I]T4-binding to αVβ3, suggesting that the association requires the binding pocket generated from the heterodimeric complex of αVβ3 and not necessarily a specific region on either monomer. To verify that the reduction in band intensity was due to specific recognition of αVβ3 by antibodies, purified αVβ3 was incubated with a monoclonal antibody to αVβ5 (P1F6) or mouse IgG prior to addition of [^{125}I]T4, neither of which influenced complex formation between the integrin and radioligand (FIG. **26**).

Example 25

T4-Stimulated MAPK Activation is Blocked by Inhibitors of Hormone Binding and of Integrin αVβ3

Nuclear translocation of phosphorylated MAPK (pERK1/2) was studied in CV-1 cells treated with physiological levels of T4 10^{-7} M total hormone concentration, 10^{-10} M free hormone) for 30 min. Consistent with results we have previously reported, T4 induced nuclear accumulation of phosphorylated MAPK in CV-1 cells within 30 min (FIG. **27**). Preincubation of CV-1 cells with the indicated concentrations of αVβ3 antagonists for 16 h reduced the ability of T4 to induce MAPK activation and translocation. Application of an RGD peptide at 10^{-8} and 10^{-7} M had a minimal effect on MAPK activation. However, 10^{-6} M RGD peptide inhibited MAPK phosphorylation by 62% compared to control cultures and activation was reduced maximally when 10^{-5} M RGD (85% reduction) and 10^{-4} M RGD (87% reduction) were present in the culture media. Addition of the nonspecific RGE peptide to the culture media had no effect on MAPK phosphorylation and nuclear translocation following T4 treatment in CV-1 cells.

Tetrac, which prevents the binding of T4 to the plasma membrane, is an effective inhibitor of T4-induced MAPK activation. When present at a concentration of 10^{-6} M with T4, tetrac reduced MAPK phosphorylation and translocation by 86% when compared to cultures treated with T4 alone (FIG. **27**). The inhibition increased to 97% when 10^{-4} M tetrac was added to the culture media for 16 h before the application of T4. Addition of αVβ3 monoclonal antibody LM609 to the culture media 16 h prior to stimulation with T4 also reduced T4-induced MAPK activation. LM609 at 0.01 and 0.001 μg/ml of culture media did not affect MAPK activation following T4 treatment. Increasing the concentration of antibody in the culture media to 0.1, 1, and 10 μg/ml reduced levels of phosphorylated MAPK found in the nuclear fractions of the cells by 29%, 80%, and 88%, respectively, when compared to cells treated with T4 alone.

CV-1 cells were transiently transfected with siRNA to αV, β3 or both αV and β3 and allowed to recover for 16 h before being placed in serum-free media. Following T4 treatment for 30 min, the cells were harvested and either nuclear protein or RNA was extracted. FIG. **28**A demonstrates the specificity of each siRNA for the target integrin subunit. CV-1 cells transfected with either the αV siRNA or both αV and β3 siRNAs showed decreased αV subunit RT-PCR products, but there was no difference in αV mRNA expression when cells were transfected with the siRNA specific for β3, or when exposed to the transfection reagent in the absence of exogenous siRNA. Similarly, cells transfected with β3 siRNA had reduced levels of β3 mRNA, but relatively unchanged levels of αV siRNA. The addition of T4 for 30 min did not alter mRNA levels for either αV or β3, regardless of the siRNA transfected into the cells.

Activated MAPK levels were measured by western blot in CV-I cells transfected with siRNAs to αV and β3, either individually or in combination (FIG. **28**B). CV-I cells treated with scrambled negative control siRNA had slightly elevated levels of T4-induced activated MAPK when compared to the parental cell line. Cells exposed to the transfection reagent alone display similar levels and patterns of MAPK phosphorylation as the non-transfected CV-1 cells. When either αV siRNA or β3 siRNA, alone or in combination, was transfected into CV-1 cells, the level of phosphorylated MAPK in vehicle-treated cultures was elevated, but the ability of T4 to induce a further elevation in activated MAPK levels was inhibited.

Example 26

Hormone-Induced Angiogenesis is Blocked by Antibody to αVβ3

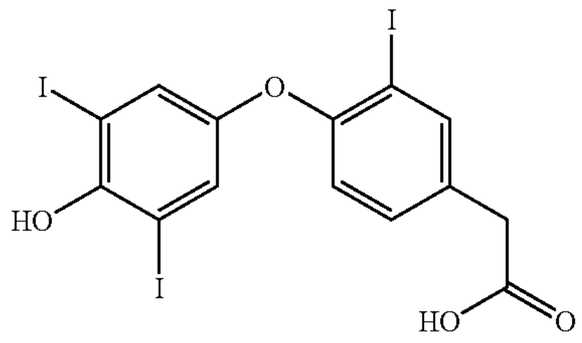
Angiogenesis is stimulated in the CAM assay by application of physiological concentrations of T4 (FIG. **29**A and summarized in FIG. **29**B). 10^{-7} M T4 placed on the CAM filter disk induced blood vessel branch formation by 2.3-fold ($P<0.001$) when compared to PBS-treated membranes. Propylthiouracil, which prevents the conversion of T4 to T3, has no effect on angiogenesis caused by T4. The addition of a monoclonal antibody, LM609 (10 μg/filter disk), directed against αVβ3, inhibited the pro-angiogenic response to T4.

Example 27

Biocompatible Polymer Conjugates of Thyroid Hormone Analogs for Short and Long-Term Delivery

Sketch 1:

Thyroid Hormone Analogs:



Triac

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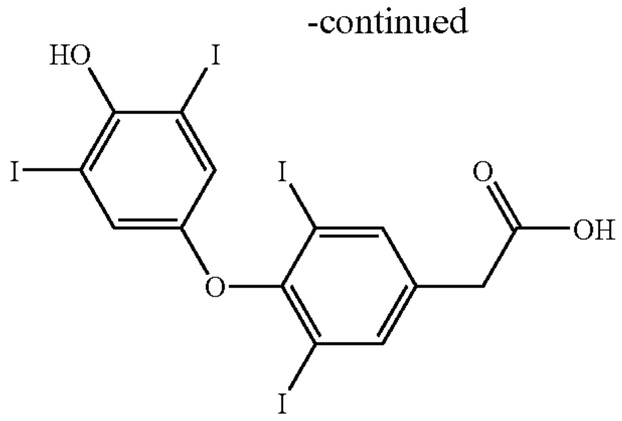
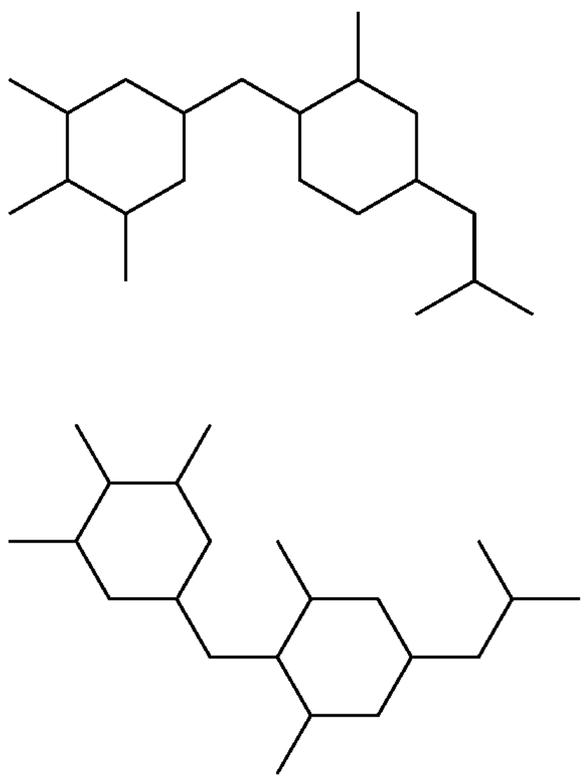


Tetrac

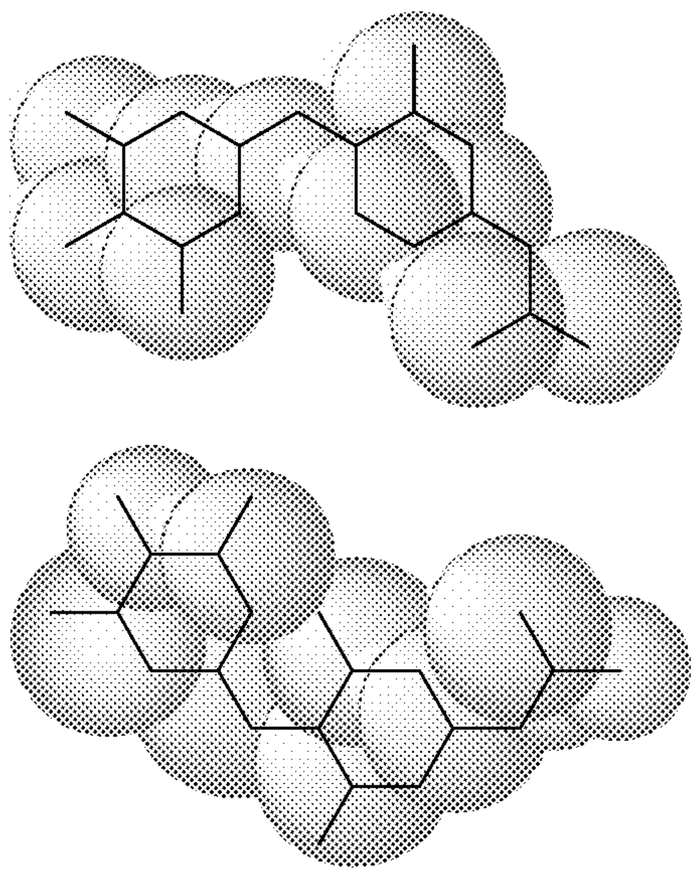
Sketch 2: Molecular Models Showing the 3 D view for Topography & Molecular Geometry in stick, ball & stick, disc and space filling models for comparative evaluation.

Stick Model: Molecules in two sets—showing molecular density in lower set.

Set 1: Triac and Tetrac (Iodines in Yellow, Oxygens in Red)

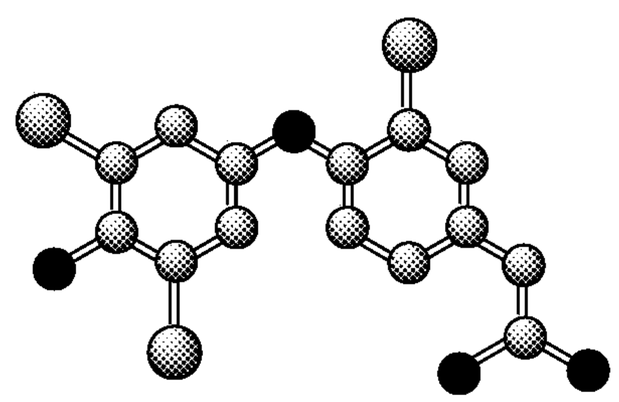


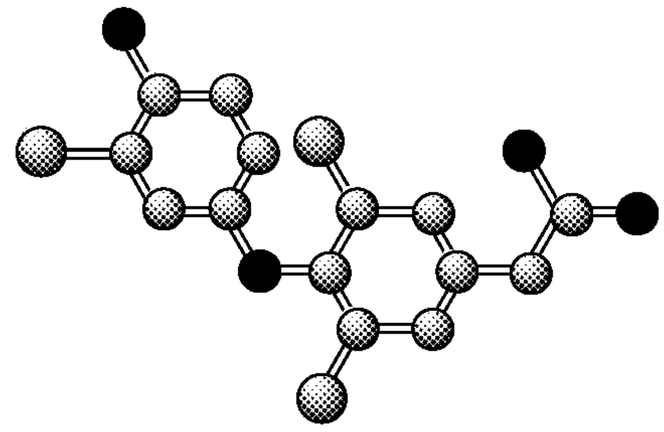
Set 2



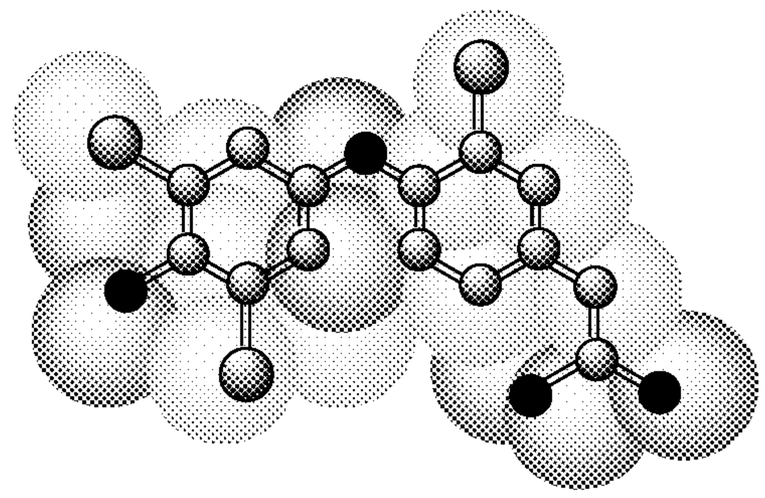
Ball & Stick Model: Triac and Tetrac—lower set showing molecular density and topographical alignment.

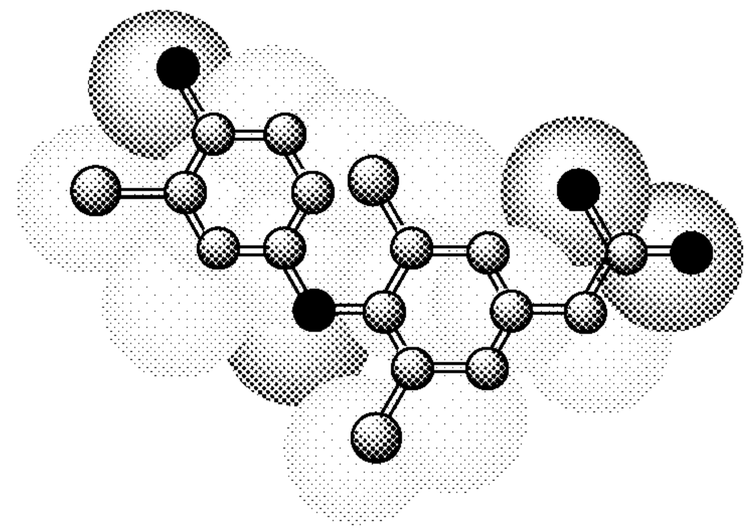
Set 1



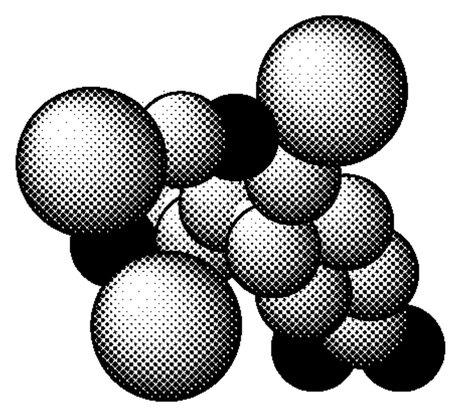


Set 2





Disc Model: Triac and Tetrac



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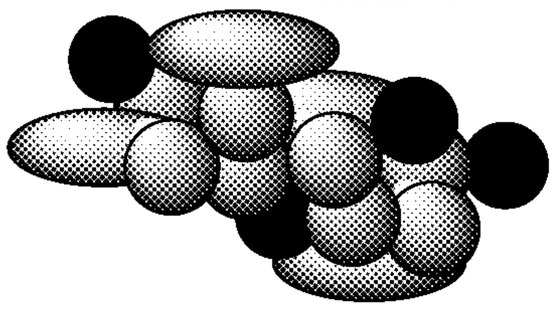
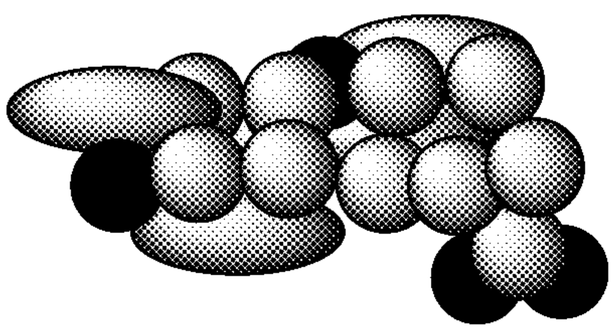
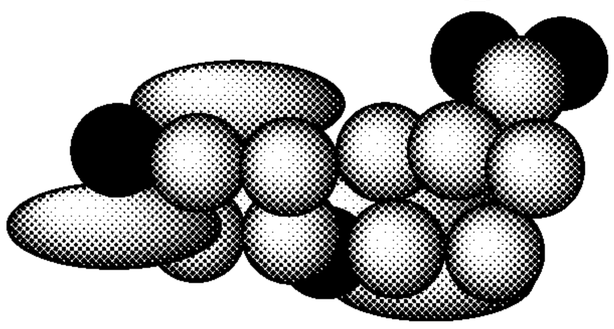


Space Filling Model: Triac and Tetrac





Thyroid Hormone Analog and Anti-Cancer Activity

The thyroid hormone metabolites Triac and Tetrac are used in treatment of thyroid cancer to enrich and as substitute therapy for its needs.

Commercially Available Thyroid Hormones & its Analogs

There are several brand names synthetic thyroid hormones available in the market including Unithroid®, Levothroid®, Synthroid® and Levoxyl®. There are generic formulations like Levo-T®, Levothyroxine Sodium® and Novothyrox®.

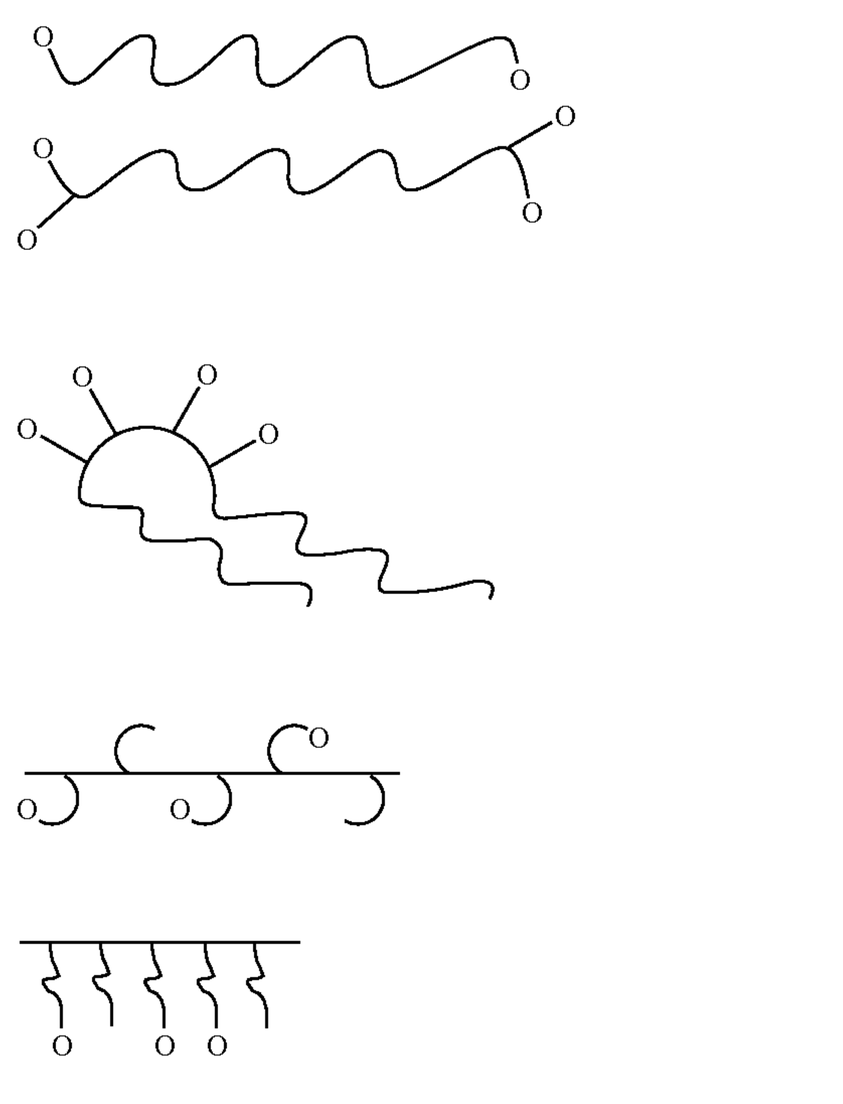
Natural Thyroid hormones are sold as food supplement in a dried and powdered form obtained from slaughtered animal's thyroid glands. This desiccated product pill may contain unwanted animal protein, improper balance of the T3 and T4 compounds and synthetic binders which is least recommended for human consumption. The ratio of T3 to T4 may vary for each batch depending on the animal's gland and has not been found to be constant for any proper delivery in human subjects. The complication in dose, delivery and availability in the system argues for a standardized, constant, limited dose and temporal release regimen for safe human consumption and other topical uses.

Thyroid Hormone Regulated Release System

As of today, no specific and standardized regimen of treatment for thyroid hormone replacement therapy is available to patients in United States. We are proposing a long term, permanent slow release system of individual thyroid constituents based on personalized needs presorted for the dose, desired activity and response profile in test systems. We will be achieving it through the polymer bound thyroid constituents delivery to the site of these products for dose defining and in a limited distribution. The proposed conjugates (FIG. **3**—a sketch) will have both short and longer term release capacity achieved through hydrolysable and non-hydrolysable characteristics. This will achieve the minimum dose level delivery for the specific cardio vascular and wound healing properties.

Sketch 3: Ordered & Random Polymer Conjugates—

Typical Polymeric Templates for Drug/API Conjugates's



O = Drug/Organic Compound/Entity being conjugated

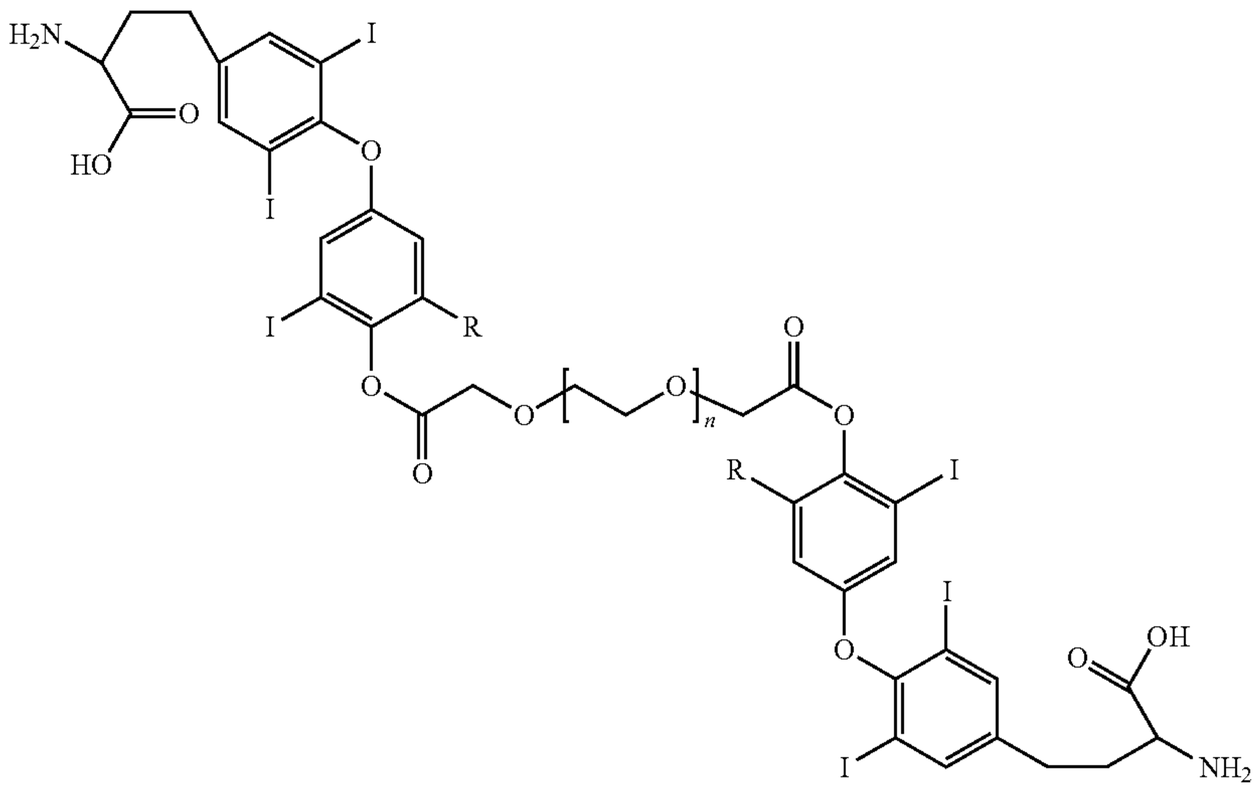
Conjugated Delivery Systems

Among the developing delivery systems, synthetic polymers conjugated chemical entities are gaining ground. A variety of synthetic, natural and biopolymeric origin side groups with efficient biodegradable backbone polymers are available and are in use in the trade. Poly alkyl glycols, polyesters, poly anhydride, poly saccharide, and poly amino acids are available for conjugation depending on the final characteristic of the conjugated product in terms of their hydrophilicity, hydrophobic nature, hydrolyses by enzymes, co-factors and biologically available acids in vivo as well as in vitro systems for the purpose.

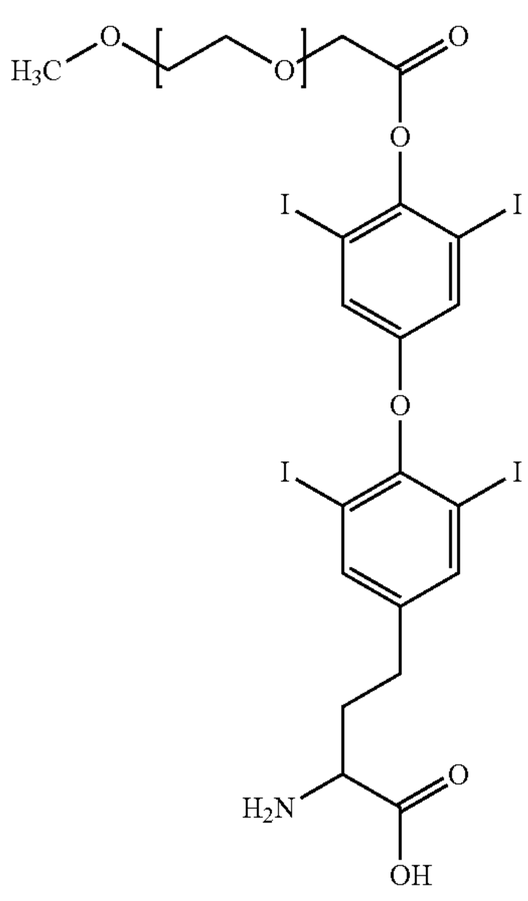
Polymer Conjugation-Synthesis & Purification

We are proposing a library of conjugated products (see FIG. **6** and Table-1) for the controlled, hydrolysable as well as non-hydrolysable polymer conjugate products including the biopolymers. A test case will evolve from each category of polymer products for their detailed study in terms of its pharmacokinetics covering delivery, transport, half-life and degradation and or erosion data. The series will also be tried to prepare as a concurrent or library design of combinatorial synthesis fitting into the similar chemical class of reaction for the substrate and varying polymers utilizing DCC, DCC/HOBt based and other water soluble reagents including placement of linker and commonly used NHS ester based synthetic strategies with a view for future developments in the polymeric conjugation combinatorial or parallel synthesis. The available facilities of the synthesizer at PRI will be utilized towards this effect and each product will be individually purified for suitability of the biological test systems.

Sketch 4: Representative Polymer Conjugate Structures from Natural, Synthetic and Polypeptide Polymer Chain.



n = chain length
R = H, Bifunctional PEG-linked-T3
R = I, Bifunctional PEG-linked-T4
PEG Based Thyroid Compounds Polymer Conjugated Delivery Systems



Methoxy-PEG-Linked-T4 Thyroid Product's Polymer Conjugate

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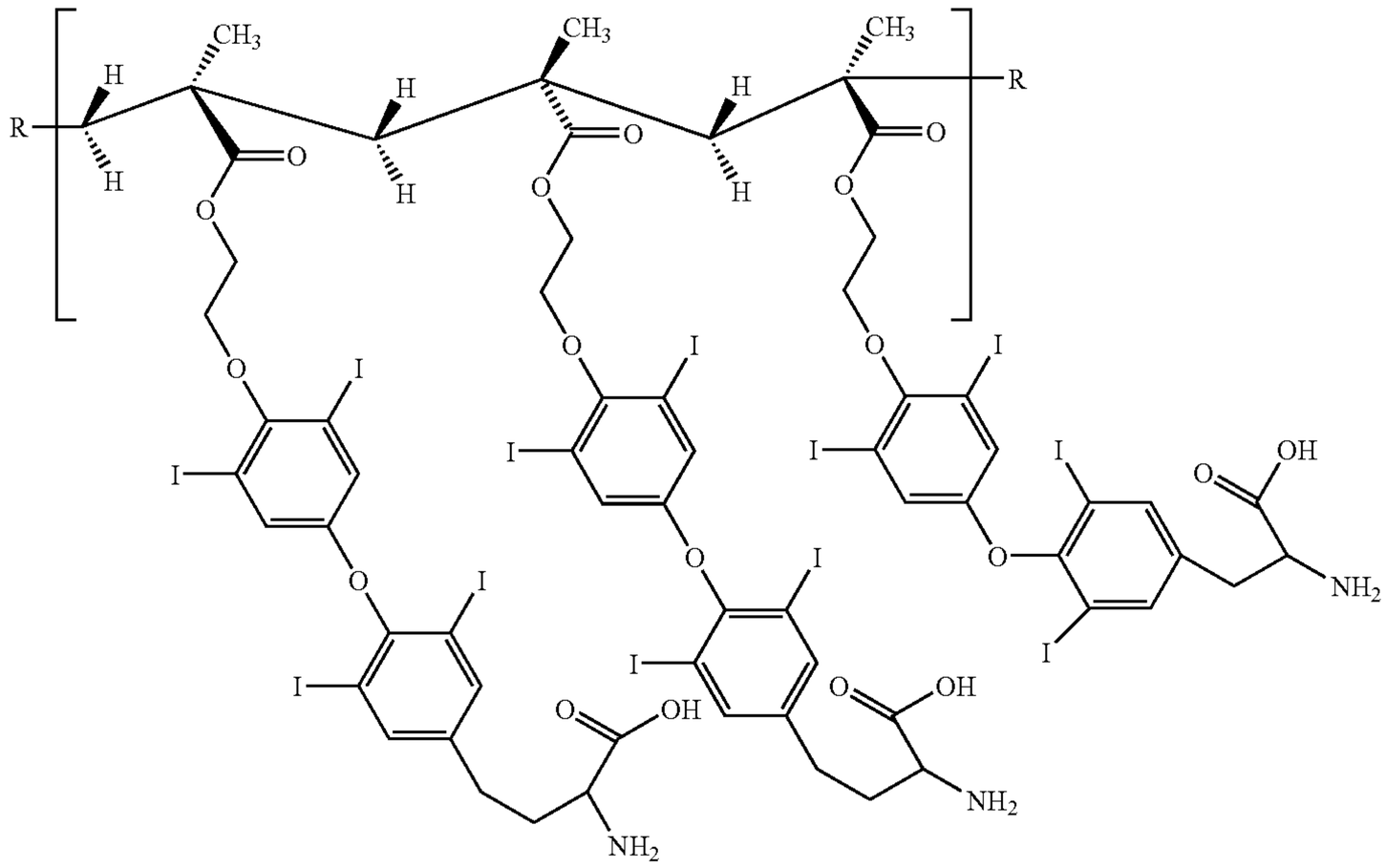
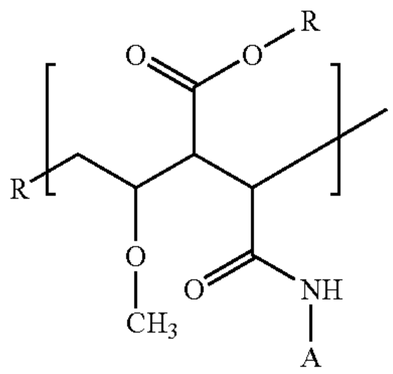


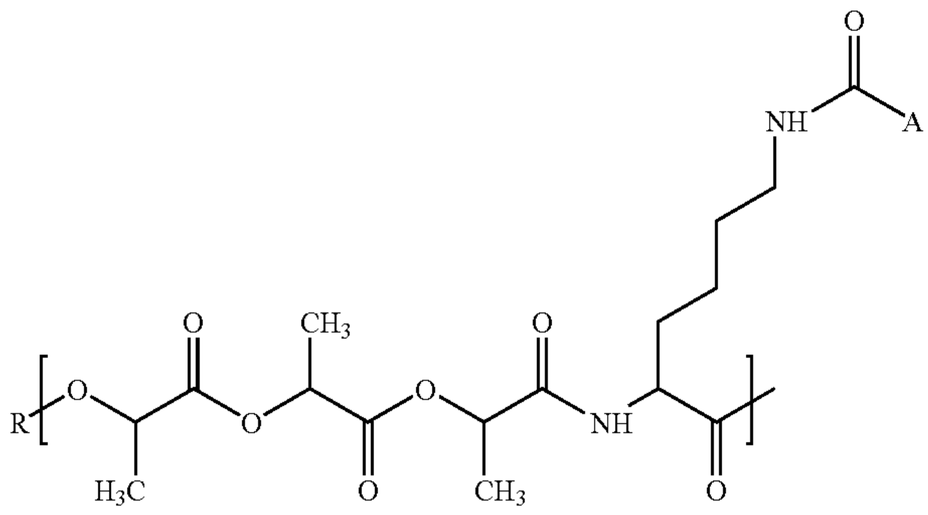
R = Repeating unit of HEMA chain

Poly-(HEMA)-Linked-T4-Conjugate



A = Thyroid Constituent (conjugated through amine end)

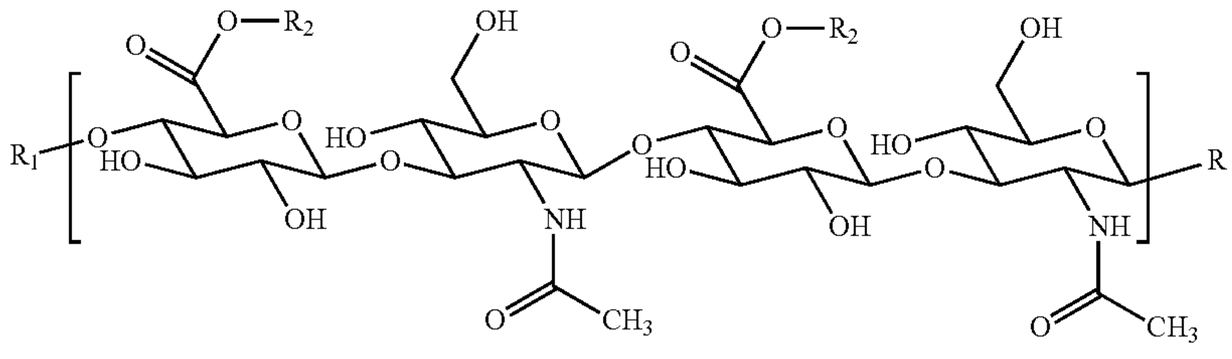
Poly-(viny-co-maleic anhydride) immobilized conjugate



R = Repeating Chain Unit

A = Thyroid Constituent (conjugated through carboxyl end)

Poly-(lactide-co-lysine) immobilized conjugate



R1 = Monomers of saccharide chain

R2 = Thyroid Constituents

Hyaluronic Acid Bound Conjugates

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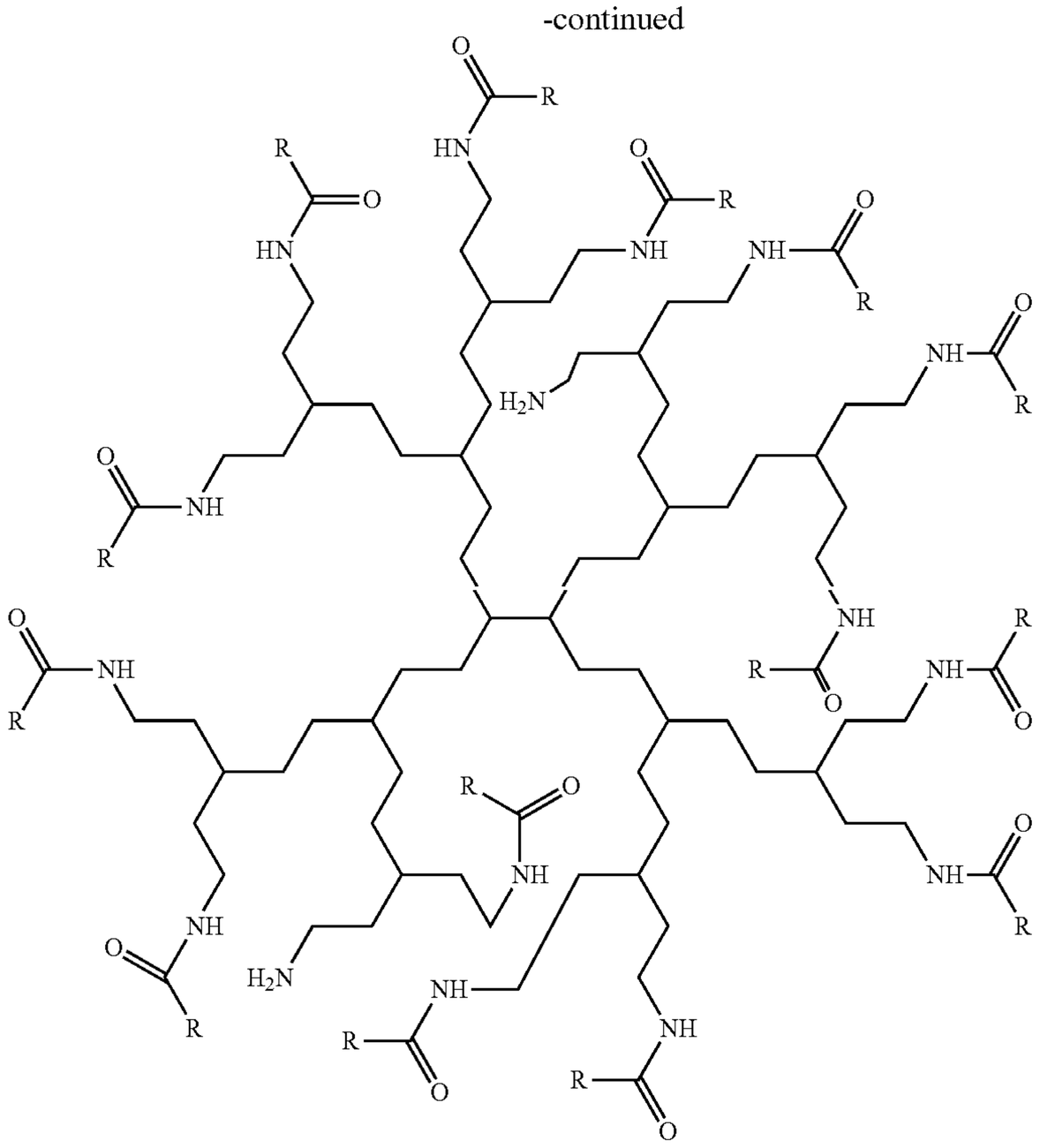
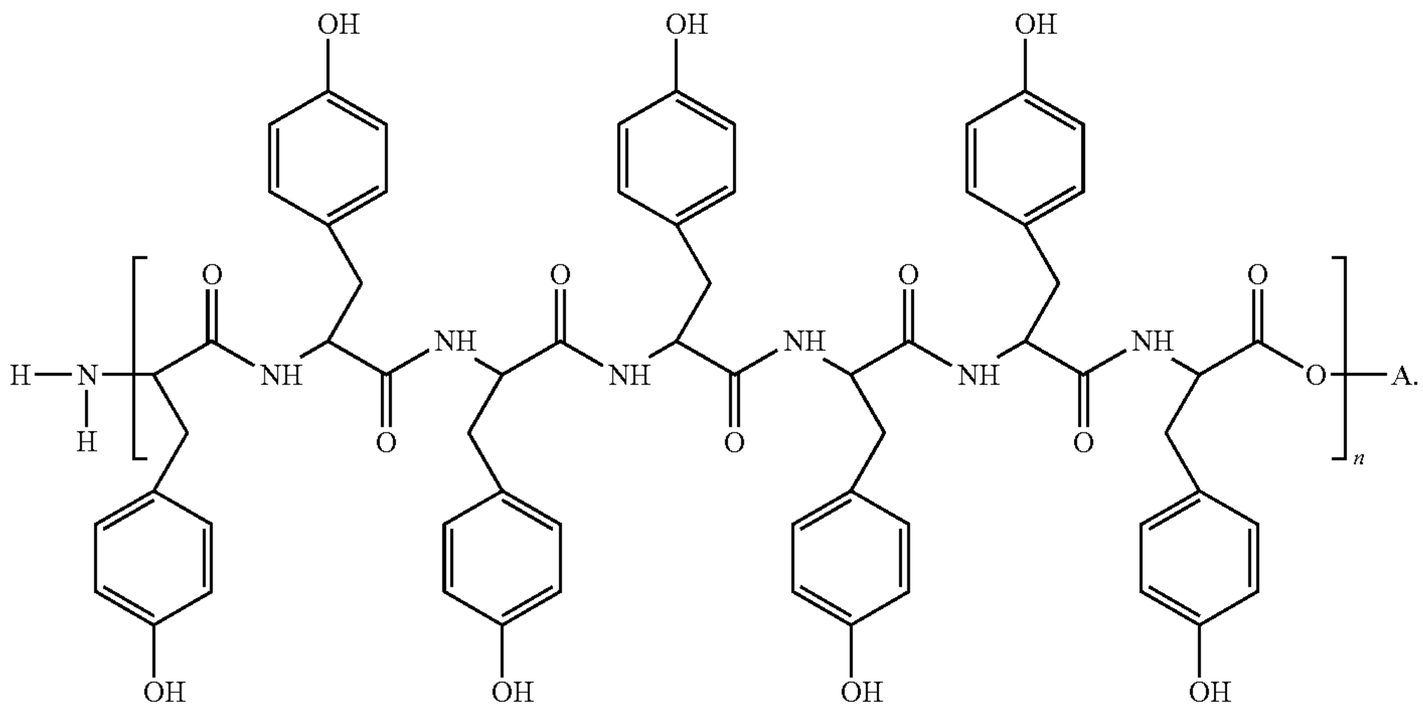


R = Thyroid Constituents T3/T4/DITPA/GC-1

Immobilized Thyroid Constituents on Multifunctional Polyamidoamine



A = Conjugated Selected Thyroid Consituent

Mobilized Thyroid Polypeptide Conjugate

Polymer Conjugates—Physical & Chemical Characterization:

A through spectro-analytical and chromatographic analysis of the polymer bound compounds will be performed using NMR (High & Low Field—Proton & Carbon, DEPT, HOMOCOSY & HETEROCOSY/HETCOR wherever applicable), IR, MS, HPLC, thermal and environmental degradation analysis for stability suitability and degradation rate profile.

Release & Stability Studies:

A detailed HPLC analyses for quantitative release analyses will be undertaken based on our established protocol for the individual thyroid products i.e., GC-1, T3, T4 and DITPA as well as Triac and Tetrac along with individual polymer and polymer conjugated product's chromatographic and spectro-analytical profile.

Polymers Compatibility Criteria for Conjugation:

Biodegradable and biocompatible polymers have been designated as probable carriers for long term and short time delivery vehicles including non hydrolysable polymeric conjugates (table 1). PEGs and PEOs are the most common hydroxyl end polymers with a wide range of molecular weights to choose for the purpose of solubility (easy carrier mode), degradation times and ease of conjugation. One end

protected Methoxy-PEGs will also be employed as a straight chain carrier capable of swelling and thereby reducing the chances of getting protein attached or stuck during the subcellular transportation. Certain copolymers of ethylene and vinyl acetate, i.e. EVAc which have exceptionally good biocompatibility, low crystallinity and hydrophobic in nature are ideal candidate for encapsulation mediated drug delivery carrier.

Polymers with demonstrated high half-life and in-system retention properties will be undertaken for conjugation purpose. Among the most common and recommended biodegradable polymers from lactic and glycolic acids will be used. The copolymers of L-lactide, and L-lysine is useful because of its availability of amine functional groups for amide bond formation and this serves as a longer lasting covalent bonding site of the carrier and transportable thyroid compound linked together through the carboxyl moiety in all the thyroid constituents.

The naturally occurring polysaccharides from cellulose, chitin, dextran, ficoll, pectin, carrageenan (all subtypes), and alginate and some of their semi-synthetic derivatives are ideal carriers due to its high biocompatibility, bio systems familiar degradation products (mono saccharide from glucose and fructose), hydrophilic nature, solubility, protein immobilization/interaction for longer term stability of the polymer matrix. This provides a shell for extra protection for polymer matrix from degradation over time and adding to the effective half life of the conjugate.

Protein & Polypeptide from serum albumin, collagen, gelatin and poly-L-lysine, poly-L-alanine, poly-L-serine are natural amino acids based drug carrier with advantage of biodegradation, biocompatibility and moderate release times of the carrier molecule. Poly-L-serine is of further interest due to its different chain derivatives, e.g., poly serine ester, poly serine imine and conventional poly serine polymeric backbone with available sites for specific covalent conjugation.

Synthetic hydrogels from methacrylate derived polymers have been frequently used in biomedical applications because of their similarity to the living tissues. The most widely used synthetic hydrogels are polymers of acrylic acid, acrylamide and 2-hydroxyethyl methacrylate (HEMA). The poly HEMA are inexpensive, biocompatible, available primary alcohol side chain elongation functionality for conjugation and fit for ocular, intraocular and other ophthalmic applications which makes them perfect drug delivery materials. The pHEMA are immune to cell attachment and provides zero cell motility which makes them an ideal candidate for internal delivery system.

Synthetic thyroid analog DITPA conjugation library design program has been achieved with the development of crude DITPA conjugated products. PVA and PEG hydrophilic polymer coupling mediated through Dicycolhexyl Carbodiimide and by other coupling reagents of hydrophilic and hydrophobic nature is under progress.

The design for the evolution of library synthesis on solid phase synthesizer is in its final stages and a model for its high throughput screening (HTS) will be put in place based on commonality of testing system and parametric criteria. The statistical analyses for the delivery time, half-life and conceived stability of the conjugates will be accumulated for the purpose of structure delivery analyses (SDA). Following is a list of intended polymer conjugates for preparation (Table 8).

TABLE 8

Library of Designated Polymer Conjugates for Possible Preparation based on Chemical Class Reactivities & Stability Data.

Sr. No.	Polymer	Properties (H Hydrolysable, NH Non Hydrolysable, RR Retarded Release)
1	PEO	H
2	m-PEG	H
3	PVA	Hydrophilic, H
4	PLLA	Hydrophilic, H
5	PGA	Hydrophilic, H
6	Poly L-Lysine	NH
7	Human Serum Albumin	Protein, NH
8	Cellulose Derivative (Carbomethoxy/ethyl/ hydroxypropyl)	Polysaccharide, RR
9	Hyaluronic Acid	Polysaccharide, RR
10	Folate Linked Cyclodextrin/Dextran	RR
11	Sarcosine/Amino Acid spaced Polymer	RR
12	Alginate/Carrageenan	Polysaccharide, RR
13	Pectin/Chitosan	Polysaccharide, RR
14	Dextran	Polysaccharide, RR
15	Collagen	Protein, NH
16	Poly amine	Aminic, NH
17	Poly aniline	Aminic, NH
18	Poly alanine	Peptidic, RR
19	Polytryptophan	Peptidic, NH/RR
20	Polytyrosine	Peptidic, NH/RR

OTHER EMBODIMENTS

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. A method for treating a condition amenable to treatment by inhibiting angiogenesis, said method comprising administering to a subject in need thereof an amount of an anti-angiogenesis agent effective for inhibiting angiogenesis in said subject, wherein said condition amenable to treatment by inhibiting angiogenesis is glioma, wherein said anti-angiogenesis agent is tetraiodothyroacetic acid (TETRAC), triiodothyroacetic acid (TRIAC), or combinations thereof, and wherein the agent effective for inhibiting angiogenesis acts at the cell surface and is conjugated via a covalent bond to a polymer selected from the group consisting of polyglycolide, polylactide, and co-polymers thereof.

2. The method of claim **1**, wherein the mode of administration of the anti-angiogenesis agent is parenteral, oral, rectal, topical, or combinations thereof.

3. The method of claim **1**, wherein the method further comprises administering one or more anti-angiogenesis therapies or chemotherapeutic agents to the subject.

4. The method of claim **1**, wherein the agent effective for inhibiting angiogenesis conjugated to a polymer is formulated into a nanoparticle, wherein said nanoparticle is less than 200 nm in size.

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